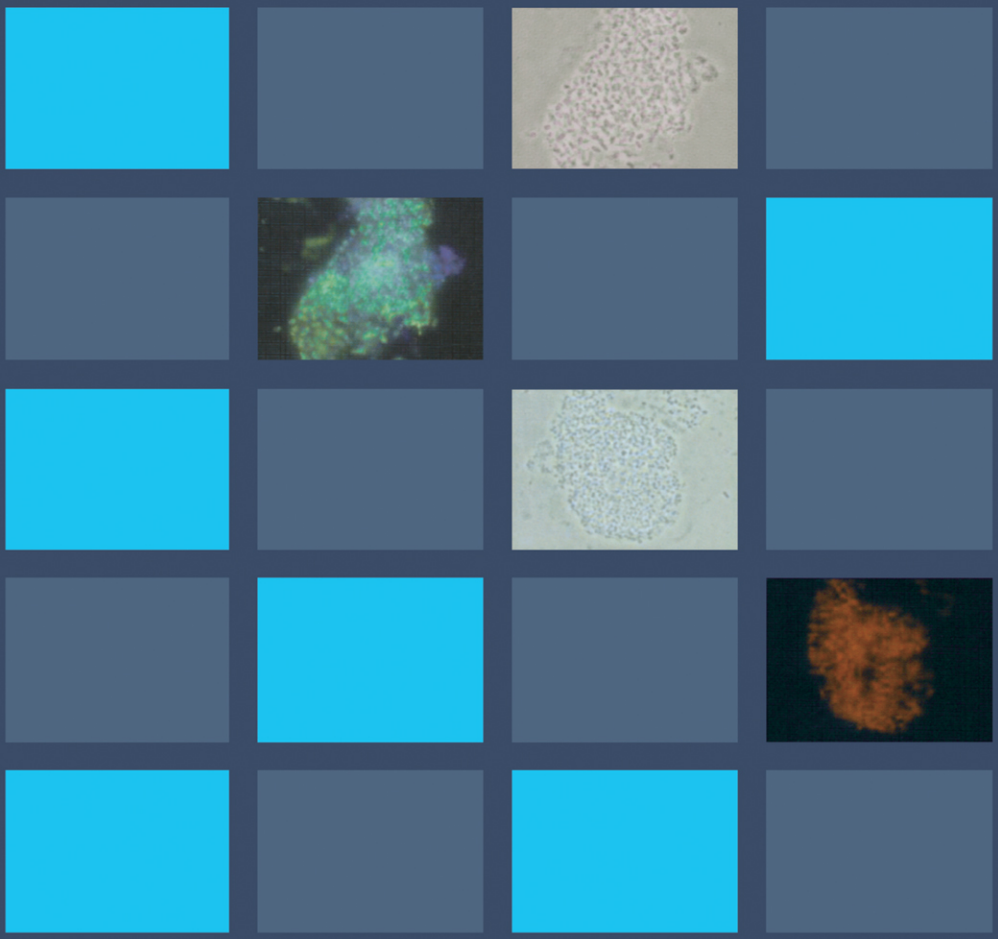
ADVANCES IN WATER AND WASTEWATER TREATMENT TECHNOLOGY

MOLECULAR TECHNOLOGY, NUTRIENT REMOVAL, SLUDGE REDUCTION AND ENVIRONMENTAL HEALTH

EDITED BY T. MATSUO, K. HANAKI, S. TAKIZAWA AND H. SATOH



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Molecular Technology, Nutrient Removal, Sludge Reduction and Environmental Health

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PREFACE

Environmental issues have become more and more critical aspects for sustainability of the earth on which we live. Global warming issue is attracting attention for global sustainability, but water environmental issues are also very critical ones and more urgent. More than one billion of people do not have good access to safe drinking water, and numerous people suffer from poor quality of water. Availability of water with enough quantity and good quality is indispensable condition for human settlement.

There is long history of engineering for improving water quality, namely water and wastewater treatment. There exist microbiological, physical and chemical processes. Among them microbiological process is one of the most reasonable processes that employs and enhances the function of bacteria in natural environment.

However, operation of biological treatment process has long been very empirical. Design and operation of process are based on the past experiences. Such experience-based technology has been satisfactory as long as conventional pollutant removal is aimed at. However, removal of wider varieties of pollutant with high efficiency and with low energy or resource consumption is nowadays required. We need to explore into details of microbial communities to satisfy such requirements. Empirical way of design and operation should be reviewed and improved under the light of advanced knowledge on complex microorganisms.

Traditional microbiology uses the approach of pure culture study. On the other hand, microorganisms work as consortium in microbiological community in the actual wastewater treatment. There exists great gap between pure culture approach and the actual process. Isolation and cultivation are basic tools in the pure culture study. However, such methods are very difficult and not always relevant in the wastewater treatment. A typical example is phosphorus removing organisms of which pure culture is hardly obtained. The second problem is that the simple summation of function of each group of the isolated microorganisms does not necessarily show the function of bacterial community in treatment process.

The appropriate approach in wastewater treatment is to examine the complex microbial community as it is. However, such research often becomes very empirical and microbial community is treated as an unknown black box. We have not had satisfactory tool to open and elucidate this box.

The rapid progress of the molecular biological techniques for analyzing microorganisms has been changing and enhancing our understanding of the complex microbial communities. This technological progress provides us new possibility of disclosing this black box. Our exploring journey is still at initial stage, but many new findings are expected in the near future.

The other important aspect of microbiology in water and wastewater treatment is the control of health-related microorganisms. Water-borne diseases are common problem in developing countries, and also critical issue in developed countries. The outbreak of new type of infectious organisms such as *Cryptospordium* since late 80's has challenged our view such that water-borne disease is the past issue in the developed countries. Molecular biological methods have enabled the detection and identification of trace amount of infectious microorganisms. Discussion in molecular level and that from the viewpoint of risk

management is being required to protect our health.

Under these background the University of Tokyo started in 1996 "Center of Excellence (COE)" project of Ministry of Education, Culture and Sports, Japan. The project is named as "Establishment and Evaluation of Advanced Water Treatment Technology Systems Using Functions of Complex Microbial Community." This project is being conducted at Department of Urban Engineering and other departments or research institutes in the University of Tokyo. This COE project is aiming at crossing the bridge between science of microbiology and practical water and wastewater treatment.

This book is based on one of the periodical symposia held under this project on March 6 – 8, 2000.

On behalf of the editors and as the former COE project leader, I wish that this book would contribute to the progress of water and wastewater treatment both from scientific and practical aspects.

Tomonori Matsuo
Professor, Toyo University
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CONTENTS

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§1 Microbial Community Analysis

For a better understanding and control of water and wastewater treatment processes, it is necessary to understand the microbial community strucure of the microorganisms used in the treatment processes. So far, the difficulty in isolating microorganisms in water treatment processes has forced water treatment engineers rather stay away from looking into the detailes of the microbial community structure. But recently developed molecular methods are expected to bring about a new methodology to look into the detailes of complex microbial community structures in water and wastewater treatment processes.

In this chapter, application of different methods especially molecular methods for different complex microbial communities related with water/wastewater treatment and environmental samples are reported. The methods employed in the collected papers are summarized as follows.

rRNA or rDNA targeted methods and reports
-FISH (Fluorescence in Situ hybridization) ..... Matsuo *et al.*, Mino *et al.*, Kurisu *et al.*
-HNPP-FISH (FISH with enhanced sensitivity by the introduction of enzymatic reactions)..... Nasu *et al.*
-PCR-DGGE (Polymerase chain reaction and denaturing gradient gel electrophoresis)..... Matsuo *et al*, Kurisu *et al.*, and Liu *et al.*
-Cloning + sequencing ..... Oyaizu *et al.*, Liu *et al.*
-ARDRA (amplified ribosomal DNA restriction analysis) ..... Tiedje *et al.*, Hashsham *et al.*

phenotypic approach
-enzymatic activity ..... Mino *et al.*
-fluorescence staining with 6CFDA and CTC..... Nasu *et al.*
-morphotype image analysis..... Hashsham *et al.*

approach to functional genes
-primer design of PHB depolymerase gene and its application ..... Sei *et al.*

combining methodology between microbial population structure and process performance
-artificial neural network approach ..... Hashsham *et al.*

The applicability of the newly developed molecular methods are diversed. Depending on the purpose of analysis and nature of the sample to be analyzed, the best suited method have to be selected. While many of the molecular methods are targeted to genotypic information which is located on ribosomal RNA and rDNA, a sequence on clomosomal DNA which is complimentary to rRNA, some researchers are also trying to include phenotypic characteristics such as morphology, enzymatic activities, and enzymatic genes.

We hope that complex microbial community structure be revieled simultaneously with its dynamics and the functions of the microorganisms, so that water treatment performance is discussed in combination with the microbial community structures.

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Observation and model analysis for the bacterial community structure of activated sludge

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Activated sludge process is a very effective way to treat wastewaters and widely used all over the world. Although we know activated sludges are mainly composed of bacteria, we do not know 'who' treats each component in wastewater and 'how' they do. Thus, both experimental and theoretical approaches have been done to elucidate community structures of activated sludge. The community structures seems to be similar and universal among different conditions in sewage treatment plants. Moreover, continuous seeding is quite important to maintain the communities and it is probably from influent.

1. FIELD OBSERVATION OF BACTERIAL COMMUNITY STRUCTURES OF ACTIVATED SLUDGES [1]

1.1. What is activated sludge ?

Activated sludge is a well known example of complex microbial community. The dominant microorganisms which constitute the activated sludge are protista and bacteria. An important index of daily maintenance of activated sludge process has been identification of species of protista, such as volticela, rotifer and so on, which could be identified by their morphologies under an ordinary optical microscope. Although activated sludges are mainly composed by bacteria, and specific capability of each bacterium is responsible for the overall performance of the processes, little is known about the bacterial communities and their roles. This is mainly because the absence of the techniques to analyze the bacterial communities.

Recently, however, it has come to be possible to analyze the bacterial constituent of activated sludge by using molecular biological techniques which are mainly based on the information of ribosomal RNA sequences. Moreover, prokaryotic classification system has greatly changed by using the rRNA information. The phylogenetic tree based on rRNA sequences for the prokaryotes, i.e., archaea and bacteria, is shown in Figure 1 [2]. Although we still do not know the details of characteristics of each bacterium, we must start from examining the bacterial constituents of activated sludge by using molecular methods. First of all, we used Fluorescent in situ Hybridization (FISH) method for which 6 oligonucleotide

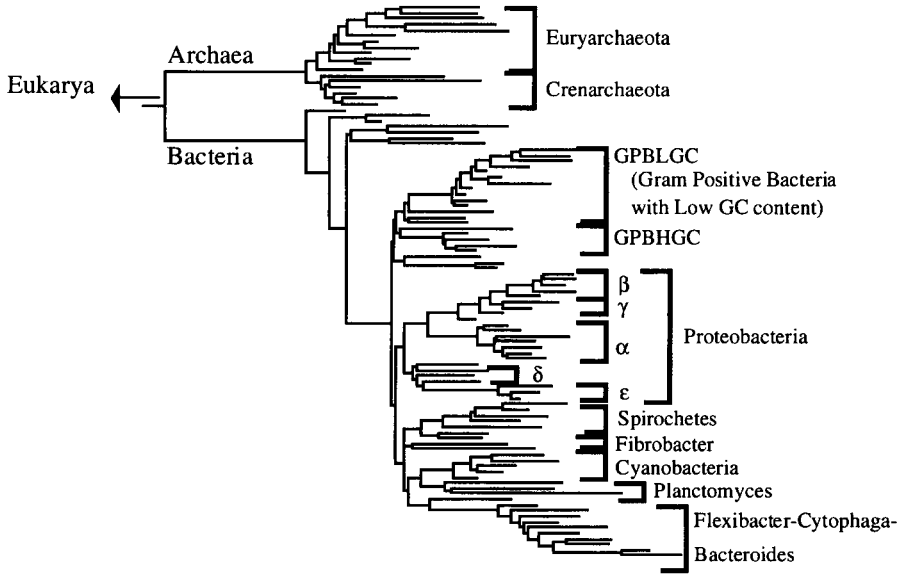

Figure 1. Phylogenetic tree of the procaryotes [2].

probes were used to clarify the composition of the bacteria at a group level in activated sludge samples. They were collected from 14 wastewater treatment plants which were operated under different conditions in Japan, because influences of operating conditions like temperature, regional background for influent water quality, sludge retention time (SRT), and hydraulic retention time (HRT) were thought to define the bacterial constituents in the activated sludges.

1.2. Methodology of the experiment

Samples

Samples were obtained from 19 activated sludge systems in 14 municipal wastewater treatment plants in Japan, as shown in Table 1. Tokyo and Saitama are located in the central area and in the temperate zone, Hokkaido is the far north and in the subfrigit zone, and Okinawa is the far south and in the subtropical zone. The samples were taken in August, 1998. They were transported in 10% ethanol at 4°C. On arrival of the samples, they were fixed with 3% paraformardehyde for 3 h and stored in a 1:1 mixture of 1xPBS and 99% ethanol at -20 °C, as described by Amann [3].

FISH

The paraformardehyde-fixed samples were immobilized on slideglasses by air drying and dehydrating by ethanol. Oligonucleotide probes were synthesized and labeled with X-rhodamine isothiocyanate (XRITC) or Cy5 by Takara Biomedicals, Japan. Group specific probes used in this study are summarized in Table 2. In situ hybridizations were performed according to Amann [3]. The percentage of formamide in the hybridization buffer was the

same as described in the references. To quantify the proportion of target species to the total bacteria, probe Eub 338 was also used together with each group specific probe. The slides were examined by confocal laser scanning microscope (TCS-NT, Leica Microsystems, Heidelberg, Germany) equipped with Ar/Kr laser.

Fluoresced areas in the images were quantified with the Q600HR image analyzing system (Leica), by thresholding at a certain intensity of fluorescence. The abundance of each specific probe group was represented by the ratio of the specific probe area to the bacterial probe area in a given field. Eight different fields were examined for each sample and probe, the maximum and the minimum ratio was discarded and the ratio of the remaining 6 fields were averaged.

Table 1
Summary of the collected Samples

Pref.	Plant	Sample	Process
Tokyo	O	Tokyo#1	Std*a
	S	Tokyo#2	〃
		Tokyo#3	〃
		Tokyo#4	〃
	NN	Tokyo#5	AO*b
	N	Tokyo#6	Std
		Tokyo#7	AO
		Tokyo#8	A2O*c
	M	Tokyo#9	Std
		Tokyo#10	〃
Saitama	FT	Saitama#1	〃
	I	Saitama#2	OD*d
	AJ	Saitama#3	OD
	AU	Saitama#4	Std
Hokkaido	FG	Hokkaido#1	〃
	AB	Hokkaido#2	〃
	B	Hokkaido#3	OD
Okinawa	MB	Okinawa#1	Std
	NG	Okinawa#2	〃

*a: Standard activated sludge process,
*b: Anaerobic-aerobic process,
*c: Anaerobic-Anoxic-Aerobic process,
*d: Oxidation Ditch process.

1.3. Results

Figure 1 summarizes the community structures in activated sludge system at the group level. α- or β- proteobacteria were the most predominant bacterial groups in all the samples. The other groups, such as Cytophagale, γ-proteobacteria and GPBHGC (High GC group of Gram positive bacteria) occupied small fractions. The ratio of the five groups against EUB probe ranged 40% to 90%. These results basically did not change even in other seasons (data not shown). The predominancy of Proteobacteria in municipal wastewater treatment plants was also reported in Germany [8,9], but some activated sludges treating industrial or synthetic wastewaters have quite different compositions [9].

In order to analyze the relationship between the community structure and plants location or processes, cluster analysis of the community structure was conducted (Figure 3). Although the

Table 2
Oligonucleotide probes used in this study

Name	Target group	Abbreviation	Sequence(5'-3')	Reference
ALF1b	α-subclass of Proteobacteria	ALF	CGTTCGCTCTGAGCCAG	[4]
BET42a	β-subclass of Proteobacteria	BET	GCCTTCCCACTTCGTTT	[4]
GAM42a	γ-subclass of Proteobacteria	GAM	GCCTTCCCACATCGTTT	[4]
HGC69a	High GC group of Gram positive bacteria	HGC	TATAGTTACCACCGCCCT	[5]
CF319a	Cytophaga-Flavobacterium	CF	TGGTCCGTGTCTCAGTAC	[6]
EUB338	Eubacteria	EUB	GCTGCCTCCCGTAGGAGT	[7]

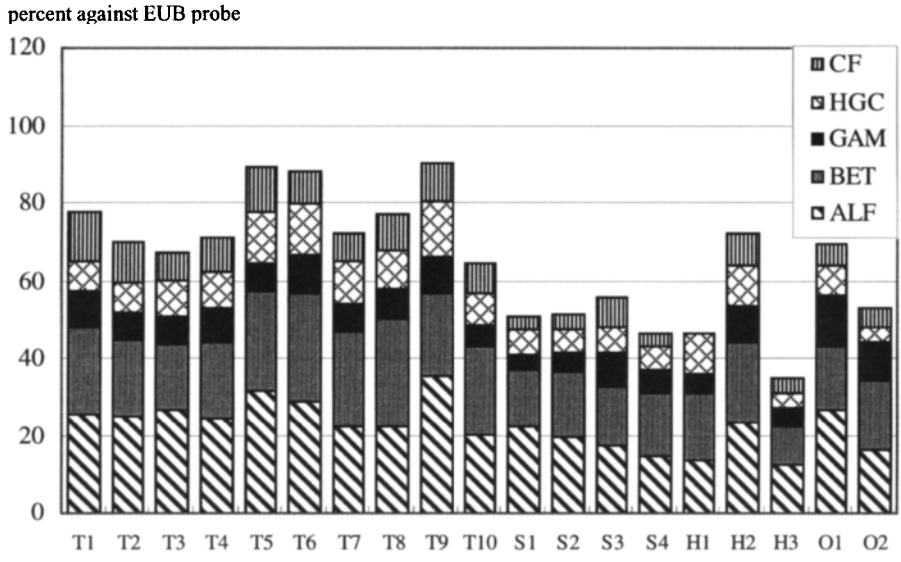

Figure 2. Microbial community structures examined by FISH with group specific probes. T1-T10:Tokyo#1-10, S1-S4: Saitama#1-4, H1-H3: Hokkaido#1-3, O1-2: Okinawa#1-2.

temperature and operating conditions were much different among the treatment process, the effect on the community structure was not clearly observed. The plants in Tokyo and Saitama are in the separate clusters, but those in Hokkaido (far north) and Okinawa (far south) distributed in both clusters, respectively. Judging from this cluster analysis, the communities in oxidation ditch (OD) process may have a unique characteristics, but the results were completely different between the four seasons (data not shown).

1.4. Summary of the experiments

(1) Proteobacteria, especially alpha and beta subclass, predominated in almost all sewage treatment plants.

(2) The community structures in the sewage treatment plants in Japan is quite similar to those in Germany at the group level.

(3) Operating conditions like temperature and process configurations does not highly affect the community structures at the group level.

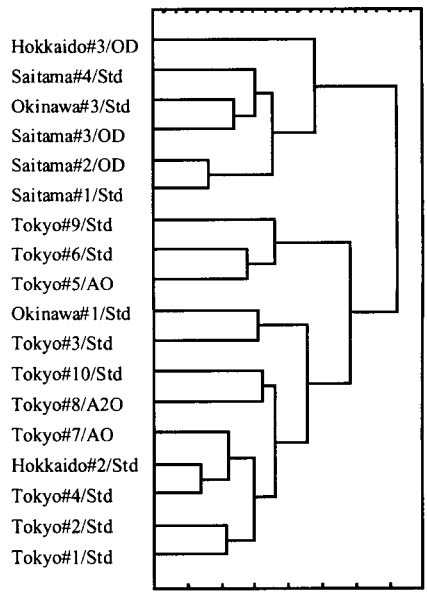

Figure 3. Cluster analysis of the composition ofthe bacterial groups, examined by FISHmethod.

2. MODEL ANALYSIS OF POSSIBILITY OF PRESERVING COMPLEX BACTERIAL COMMUNITY IN CHEMOSTAT CULTIVATION OF BIOMASS [10]

2.1. Presence of continuous seeding

Based on the observation in the previous section, it is reasonably assumed that more than two different bacteria should be present at the same time in the reactor, even in the fixed operating condition of HRT of chemostat operation in a continuously stirred tank reactor (CSTR). It is, however, not so much obvious that two different bacteria can survive under one HRT condition, if a homogeneous cultivating condition is assumed in the reactor. Basic mass balance equations of growth of biomass (bacteria) in the CSTR is presented as Eq. 1.

$$\begin{cases} \dfrac{dX}{dt} = \dfrac{X_{in} - X}{\tau} + \mu \dfrac{S}{K+S} X - bX = 0 \\ \dfrac{dS}{dt} = \dfrac{S_{in} - S}{\tau} - \dfrac{1}{Y} \mu \dfrac{S}{K+S} X = 0 \end{cases} \tag{1}$$

Where,
X : biomass concentration in the reactor,
S : substrate concentration in the reactor,
Xin : biomass concentration of raw sewage,
Sin : substrate concentration of raw sewage,
τ : hydraulic retention time of the reactor,
b : biomass decay rate,
Y : yield coefficient,
μ : maximum growth rate,
K : saturation constant in the Monod expression of growth rate.

In the relationship of Eq. (1), the necessary condition of the washout of the biomass from the system is that the biomass concentration x should come to be zero at the stage of steady state. Then the necessary condition for this situation is expressed as Eq. (2).

$$\frac{X_{in}}{\tau} = 0 \tag{2}$$

But it is clear that this condition dose never satisfy the original assumption of the presence of continuous seed supply to the system. This relation concludes that every species of bacteria which is supplied continuously should always be preserved in the system.

2.2. Absence of continuous seeding

For the condition of no entry of bacteria from outside, in which Xin is assumed to be zero, then the Eq. (1) is rewritten as Eq. (3) in case of preserving the two different type of bacteria.

$$\begin{cases} \dfrac{dX_1}{dt} = \dfrac{0 - X_1}{\tau} + \mu_1 \dfrac{S}{K_1 + S} X_1 - b_1 X_1 \\ \dfrac{dX_2}{dt} = \dfrac{0 - X_2}{\tau} + \mu_2 \dfrac{S}{K_2 + S} X_2 - b_2 X_2 \\ \dfrac{dS}{dt} = \dfrac{S_{in} - S}{\tau} - \dfrac{1}{Y_1} \mu_1 \dfrac{S}{K_1 + S} X_1 - \dfrac{1}{Y_2} \mu_2 \dfrac{S}{K_2 + S} X_2 \end{cases} \quad (3)$$

Where,
X_1, X_2 : biomass concentrations of species 1 and 2, respectively,
S : substrate concentration in the reactor,
S_{in}: substrate concentration of raw sewage,
τ: hydraulic retention time of reaction,
b_1, b_2 : biomass decay rate of species 1 and 2, respectively,
Y_1, Y_2: yield coefficient of species 1 and 2, respectively,
μ_1, μ_2: maximum growth rate of species 1 and species 2, respectively, and
K_1, K_2,: saturation constant in the Monod expression of growth of species 1 and 2, respectively.

Under the steady state conditions of $dX_1/dt=0$, $dX_2/dt=0$, and constant substrate concentration, the solution of Eq. (3) for the relationship of growth parameters of two different species is obtained as Eq. (4). Without continuous feeding of source of the specific bacteria, only a pair of bacteria which satisfies very strict relationship described as Eq. (4) can survive. Otherwise, only one of the bacteria can survive and the other one is washed out because the SRT is too short to the bacterium which has slower specific growth rate.

$$\frac{K_1(b_1\tau + 1)}{\mu_1\tau - (b_1\tau + 1)} = \frac{K_2(b_2\tau + 1)}{\mu_2\tau - (b_2\tau + 1)} \quad (4)$$

This requirement yields the relation of Eq. (4) for the parameters of specific growth rate and half rate constant, and hydraulic retention time. The above mentioned relation means that the microbial community structure with two or more growth parameters should be strictly limited in a very specialized group of microorganisms which satisfy the relationship of Eq. (4). If bacteria are supplied continuously to the system, it is possible to keep both bacteria in the reactor as examined in Eq. (2), as shown in the previous section. From this analogous explanation, it is assumed that the very rich microbial diversity observed in the activated sludge is possibly caused by continuous feeding from raw sewage or continuous supply from atmospheric air.

2.3. Conclusions from theoretical model analysis

(1) Even in the chemostat operation of CSTR, every species of bacteria can be preserved in the reactor, if the seed of the bacteria is continuously supplied to the system.
(2) In case of the no seed supply, only very limited group of species can survive in the

system.
(3) In the usual activated sludge operation with settling unit operation, sludge operation with settling process, the bacterial community might be much more complex than the simple chemostat operation. It is assumed, however, that the continuous feeding of seed bacteria outside of the system, is essential to keep the complex bacterial community structure in the system.
(4) The possible sources of the seed bacteria are thought to be raw sewage and/or atmospheric air, which are supplied continuously from outside of the system.

3. EXPERIMENTAL INVESTIGATION ON SOURCES OF BACTERIA FOR COMPLEX COMMUNITY STRUCTURE OF ACTIVATED SLUDGE [11]

3.1. Introduction and Experimental Setup

To identify the origin of the bacterial communities in the activated sludge, denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rDNA partial sequences (PCR-DGGE) was applied to the activated sludge samples cultivated in the batch type operation using raw sewage and synthetic sewage with atmospheric air and filtered atmospheric air. A simple experimental study was carried out using sequencing batch reactor with sedimentation period as shown in Figure 4. In the experimental conditions, three kinds of influents which were synthetic wastewater, pasteurized raw sewage by autoclave and non pasteurized raw sewage were used, and two conditions of supplied air which were filtered one by 0.2 μm membrane filter and non filtered atmospheric air from outside were used. The experimental condition of influents and air, and the operating condition of the treatment system is summarized and listed in Table 3 and 4, respectively.

3.2. Analytical method for the community structures

Suspended samples were washed with TE buffer and genomic DNA was extracted with benzyl chloride followed by ethanol precipitation as described by Zhu *et al.* [12]. PCR-DGGE was performed according to Muyzer *et al.*[13]. Primers complementary to conserved regions were used to amplify a 194-bp fragment of the 16S rDNA corresponding to nucleotides 341 to 534 in the *E.coli* sequence[13]. Denaturing gradient gels were prepared by using Model 475 gradient delivery system (Bio-Rad Laboratories, Hercules, Calif.). The gels were run by D Code system (Bio-Rad) for 300 min at 60 ^{o}C and 130V. After completion of electrophoresis, the gels were stained with Vistra Green (Amersham Pharmacia Biotech, Tokyo, Japan) by spreading the staining solution on the gel for 15 minutes, and documented by fluorescent image scanner (Fluorimager 595, Molecular Dynamics, Sunnyvale, Calif.).

3.3. Experimental Results

In case of synthetic wastewater and filtered air (condition 'e' in Table 3), there is no accumulation of biomass in the system so far as more than two weeks operation. Thus, this experimental set-up was proven to avoid any bacterial contamination from outside.

When synthetic wastewater and non filtered air were applied (condition 'a'), presence of

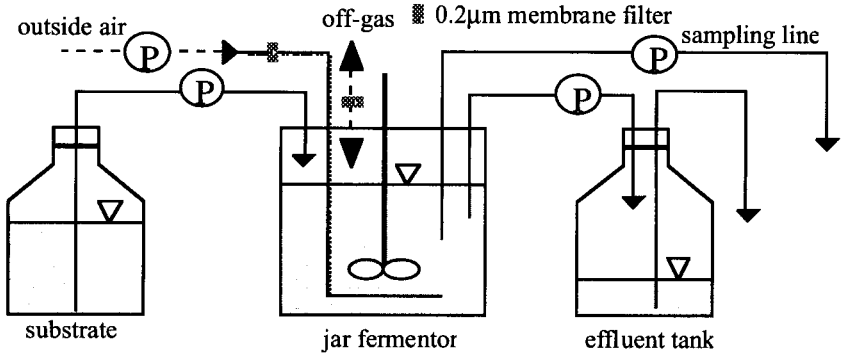

Figure 4. Experimental set-up for the sequencing batch operation reactor.

Table 3
Experimental conditions of influents and air

influent	aeration	
	filtered air	outside air
synthetic wastewater	e	a
autoclaved sewage	-	b
raw sewage	c	d

Table 4
Operating condition of the sequencing batch reactor

reactor volume	1.5L
batch cycle	8h
HRT	0.95d
SRT	7.0d
aeration rate	2L/min

organisms is clearly observed in the reactor and they created settlable flocks after 3 days operation. The bacterial community profiles is shown in Figure 5. However, these bacteria were completely different from the bacteria observed in other cases (Figure 6, 'a' vs 'b, c,d'). When raw sewage is used, almost same DGGE profile is obtained in both cases of filtered and non filtered air (Figure 6, 'c' and 'd'). These results indicated that bacteria from atmospheric air could not compete to those in raw sewage. In addition, the raw sewage and samples from the reactor fed with the sewage (condition 'c' and 'd'), and the raw sewage and the activated sludge of the sewage treatment plant fed with the same sewage, shared some same DGGE bands, which implied they have the same bacterial strains. However, their DGGE band profiles were basically different.

3.4. Conclusions of the Experiment

(1) The bacteria from atmospheric air were able to produce settlable flocks but not able to compete so much to those in raw sewage.
(2) Raw sewage was the main source of bacteria for the communities of activated sludge.
(3) The activated sludge still had different bacterial community structures from raw sewage.

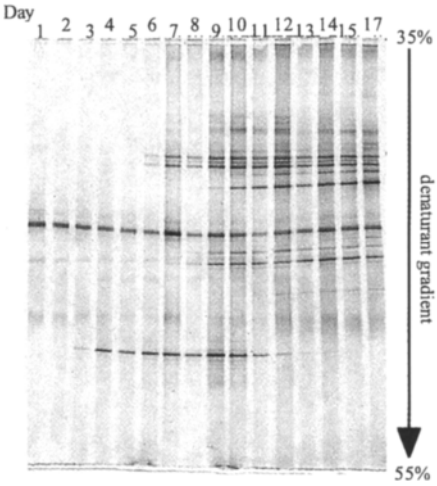

Figure 5. Daily change of the community structure analyzed by using PCR-DGGE method. Sample: condition 'a', i.e. synthetic wastewater aerated with outside air.

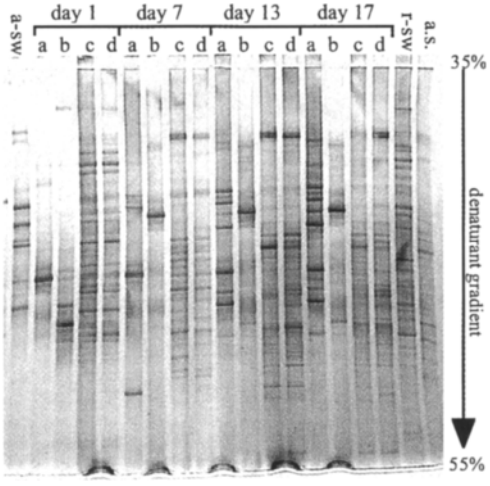

Figure 6. Comparison of the community structures and their change among operating conditions 'a' to 'd'. 'a-sw': autoclaved sewage, 'r-sw' : raw sewage, and a.s. : activated sludge taken from a pilot plant fed with the same sewage.

4. CONCLUSIONS

(1) Actual activated sludges of broad range of operating conditions have rather similar bacterial group composition in spite of the difference of regional and climate conditions.
(2) In the suspended biomass growth, the theoretical analysis predicts that it is not so easy to keep microorganisms which have different growth rate parameters in the CSTR. To preserve the diversity of bacterial community, it is required to continuously supply seeds of them from outside of the system.
(3) The most possible source of bacteria for activated sludges is raw sewage. If the main source of bacteria in the activated sludge is raw sewage, it is reasonably confirmed that the above conclusion (1) is acceptable, because the source of bacteria come mainly from human wastes and human wastes are homogeneous around regions and locations all over the world.

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REFERENCES

1. Maidak, B. L., G. J. Olsen, N. Larsen, R. Overbeek, M. J. McCaughey, and C. R. Woese. (1997) Nucleic Acids Res. 25: 109-111.
2. Akiyama, T., H. Satoh, T. Mino, and T. Matsuo. (2000) J. Japan Soc. Wat. Environ., 23: 271-278 (in Japanese).
3. Amann, R. I. (1995) p. 3.3.6.1-15. In A. D. I. Akkermans, J. D. van Elsas, and F. J. de Bruijn (ed.), Molecular microbial ecology manual, Kluwer, Dordrecht, The Netherlands.
4. Manz, W., R. Amann, W. Ludwig, M. Wagner, and K.-H. Schleifer. (1992) System. Appl. Microbiol. 15: 593-600.
5. Roller, C., M. Wagner, R. Amann, W. Ludwig, and K.-H. Schleifer. (1994) Microbiology, 140: 2849-2858.
6. Manz, W., R. Amann, W. Ludwig, M. Vancanneyt, and K.-H. Sheleifer. (1996) Microbiology 142: 1097-1106.
7. Amann, R. I., L. Krumholz, and D. A. Stahl. (1990) J. Bacteriol. 172: 762-770.
8. Wagner, M., R. Amann, H. Lemmer, and K.-H. Schleifer. (1993) Appl. Environ. Microbiol. 59: 1520-1525.
9. Manz, W., M. Wagner, R. Amann, and K.-H. Schleifer. (1994) Wat. Res. 28: 1715-1723.
10. Inoue, N. (1999) Master Dissertation in the Univ. of Tokyo (in Japanese).
11. Tanaka, M. (2000) Graduation Dissertation in the Univ. of Tokyo (in Japanese).
12. Zhu, H., F. Qu, and L. H. Zhu. (1993) Nucleic Acids Res. 21: 5279-5280.
13. Muyzer, G., E. C. Waal, and A. G. Uitterlinden. (1993) Appl. Environ. Microbiol. 59: 695-700.

Stability, persistence and resilience in anaerobic reactors: a community unveiled

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The stability of function and its relationship to microbial community structure was studied in anaerobic methanogenic reactors fed glucose over a 600 day period, and in reactors that experienced a shock-load of glucose. In the first case, the *Bacteria* populations as measured by 16S rDNA analysis varied in a chaotic manner throughout the period while function as measured by pH and effluent COD remain stable. Hence, many combinations of populations produced common function. Two different communities, one of which was high in Spirochetes, but both functioning equally well in digesting glucose were fed a pulse of glucose 10-fold higher than the normal feed. The high spirochete community showed more resistance and resilience of function, had less diversity, and its community returned to its original composition. Its superior performance was attributed to community flexibility, i.e. minor populations which accommodate the shock-loading, while the more stable low spirochete community did not tolerate the shock-loading as well.

1. INTRODUCTION

Reliable, efficient and rapid processing of organic matter is the goal of transferring natural microbial communities to reactors for processing higher amounts of carbon. The environment can be better controlled in reactors and the assumption is that this control also helps maintain and stabilize microbial populations important to efficient function. We have studied two questions concerning community stability with anaerobic reactors fed one carbon substrate, glucose. The first question is how constant is the microbial community in a reactor maintained under constant conditions for over 1500 days, and the second question is how fast and reliably do communities return to their normal function and composition after they are subject to perturbation. In this case the perturbation was a shock-load of glucose.

Anaerobic communities are particularly interesting for microbial stability studies because substrate processing requires a food chain, a series of at least three guilds of microbes specialized in the steps converting carbohydrate to methane and carbon dioxide. They must operate in coordination for the community to prosper and the function to be efficient.

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Secondly, this ecosystem operates near the thermodynamic limits of stability and hence is particularly sensitive to perturbations. Furthermore, the members of these communities are thought to be well understood, particularly with regard to their metabolic pathways, requirements, and identity.

This paper summarizes highlights of work presented in more detail elsewhere [1-3].

2. MATERIALS AND METHODS

Studies on Stability and Persistence. A 1.5-liter continuously stirred tank reactor was operated anaerobically at 35°C for 1505 days [2,4]. Glucose was supplied in a periodic cycle: 16g/liter on one day followed by no glucose feeding the second day. A constant dilution rate of 0.1 day $^{-1}$ was maintained. Steady-state function as measured by effluent COD, volatile fatty acids concentration, methane production, and pH. Steady-state was achieved after 400 days.

The microbial community structure was analyzed by amplified ribosomal DNA restriction analysis (ARDRA) [5]. Genomic DNA was extracted at seven sampling periods between day 900 and 1500. 16S rDNA was amplified from the extracted DNA using both *Bacteria* and *Archaea* primers. Amplicons were cloned with a TA cloning kit, transformed into *E. coli*, reamplified, and the appropriate sized amplicons were digested simultaneously with two restriction enzymes, *Hae*III, and *Hha*I. The restriction fragments from each clone were separated by electrophoresis on a 3.5% MetaPhor agarose gel. Restriction patterns were normalized and compared with Gel Compare software. Pattern clustering was done by the unweighted pair group method with averages with application of the Dice coefficient. Thirty-six ARDRA patterns were analyzed for each sample and frequencies were calculated as percentages of the clones showing the same operational taxonomic unit (OTU). OTUs found most frequently were sequenced, aligned and their nearest neighbors were identified using the ribosomal database (RDP).

Studies on Response to Perturbations. In these experiments, we studied the response of two very different anaerobic, glucose-fed communities to perturbation. One community was comprised of 40% to 50% spirochetes and the other was comprised of more diverse organisms, but typical of those more commonly found in anaerobic digesters. In this case, the reactor volumes were smaller, 250-360 ml, so that we could manage 4 replicate reactors of each community. Both reactor sets were subjected to an instantaneous 20-fold increase in glucose concentration. The response to this shock-loading of substrate was followed by fatty acid analysis, ARDRA, morphological analysis (image analysis) of the populations, T-RFLP, and rRNA quantitative hybridization of certain populations. More detail on the reactor operation and analyses is described in a companion manuscript in this volume [6].

3. RESULTS AND DISCUSSION

Stability and Persistence. The methanogenic reactor exhibited constant performance as measured by effluent pH and COD removal from day 400 through day 1490 (Figure 1). The pH ranged from 6.85 to 7.07 and the COD removal was between 89% and 99% until the reactor began to fail on day 1491.

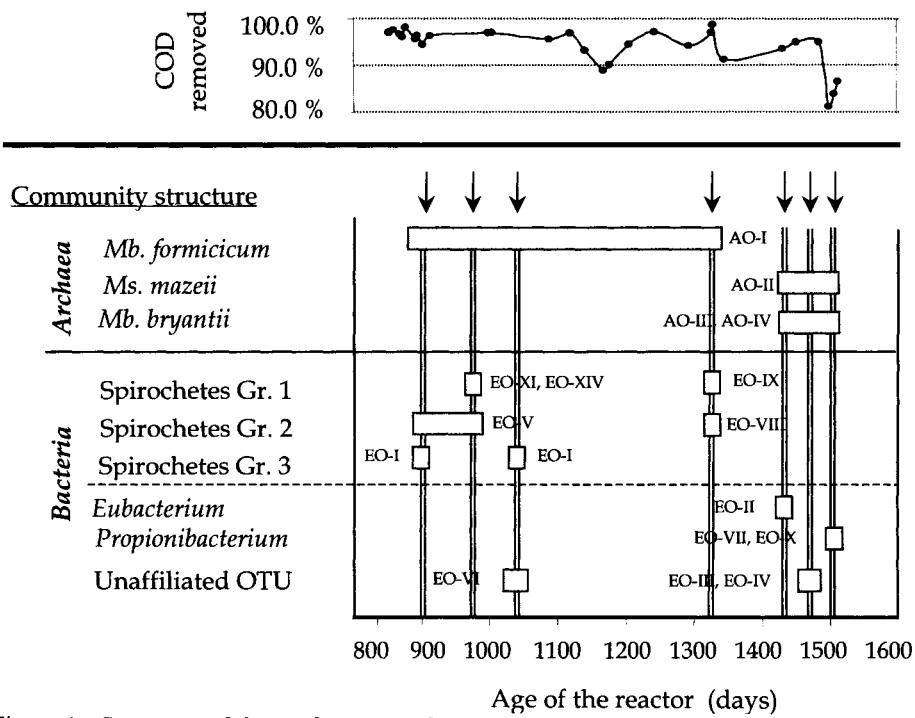

Figure 1. Summary of the performance (function) and dominant populations in an anaerobic reactor maintained under constant conditions for over 1500 days. Redrawn from Fernandez *et al.*[2].

Changes in *Archaea* populations were observed by comparing ARDRA pattern frequencies over the 600 day time period shown in Figure 1. The dominant *Archaea* population was rather constant from day 900 through day 1325, but then abruptly shifted to a completely different community at day 1437 and maintained this community through the last days of stable performance and even for the 15 days during the reactor failure. Organisms closely related to *Methanobacterium formicicum* dominated the first period and *Methanosarcina mazei* and *Methanobacterium bryantii* dominated the later period.

The *Bacteria* community was much more variable than the *Archaea* community (Figure 2). Rapid population shifts occurred and no long periods of particular OTU persistence could be distinguished. Furthermore, almost no trends in population succession could be detected, even for an interval as short as 33 days. The diversity as measured by ARDRA profile richness was also chaotic. The total number of OTUs at each sampling point range between 8 and 24. The OTUs that were sequenced were not closely related to other known organisms. They represented the four clusters shown in Figure 1. The most dominant group of organisms recovered by the rDNA method were members of the Spirochetes. The other dominant organisms are affiliated with *Eubacterium, Propionibacterium* and one unusual OTU that had less than 80% similarity to any known organism. While the successional pattern of *Bacteria*

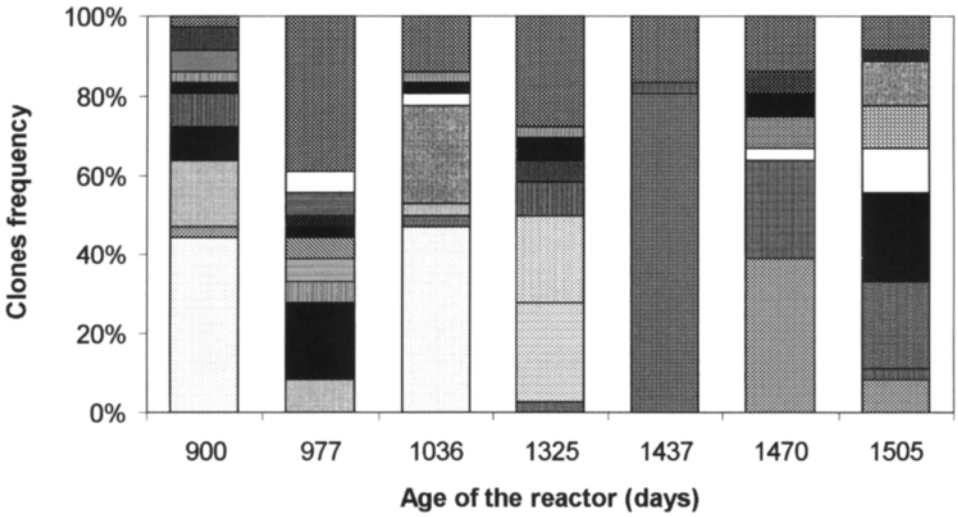

Figure 2. Variation in dominant *Bacteria* over time in a stable anaerobic reactor. Each pattern represents a different OTU and the bar size represents its frequency in the 16S rDNA clone library. Redrawn from Fernandez *et al.*[2].

was chaotic, the first period was dominated by different groups of Spirochetes. Hence, there was cycling of dominance within the Spirochetes.

Ecosystem stability often means different things to different disciplines. To some, stability refers to function and to others, it implies constant composition of the community. In this paper we are referring to the later as persistence. A difficulty in limiting the concept of stability to function is that information about the populations is often ignored, however, it is the properties of the populations, particularly their fitness parameters, that are the heart of the information needed to more intelligently manage communities. The results from our work indicate that an extremely dynamic community sustains a functionally stable system. Dominant members change dramatically, even over periods as short of 3.3 retention times. These results also indicate that functional parameters, like pH and COD, are inadequate to reveal community structure variation.

Phylogenetic analysis revealed that the genetic changes detected by ARDRA suggested metabolic variation in the community. However, if similar physiology is assumed for phylogenetically related taxa, the metabolic variation was less dramatic than were the genetic changes. Since the anaerobic food chains involve close interactions between different guilds, we expected that there would be a correlation between any changes in the *Bacteria* and *Archaea* communities. This was only moderately apparent in our study. The most obvious was that the Spirochetes occurred during the period when the Archaea were dominated by *Methanobacterium formicicum* but disappeared in the later period when the methanogen community shifted. We do not know if this relationship is causal and if so which component would have initiated the change (Figure 1).

Response to Perturbations. Ecologists quantify several parameters of stability as described in Figure 3. The two main parameters commonly measured in this amplification

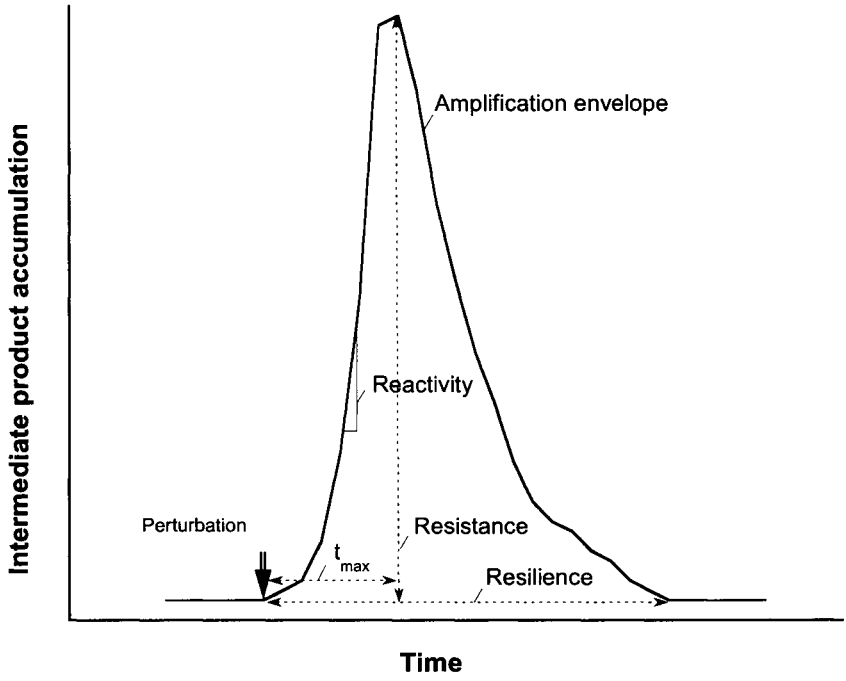

Figure 3. Ecological parameters used to describe components of stability.

envelope are resistance, which is the degree of deviation from the steady state, and the resilience, which is the time required for the community to return to its normal state[7,8]. We measured the fatty acids produced in response to the shock-loading for the two communities and evaluated their resistance and resilience. Another component that can be useful in characterizing stability is the reactivity which is defined by the maximum slope of the rising limb of the envelope.

All reactors performed uniformly for the 2 weeks before the shock-loading indicating replication of function without replication of community structure, consistent with the finding in the previous long-term study.

The low spirochete reactors (LS) had less resistance and resilience to perturbation since the fatty acid accumulation was higher and took longer for the fatty acids to return to steady-state levels than for the high spirochete reactors (HS) (Figure 4). The low spirochete reactor, however, showed more diversity both by image analysis and ARDRA showing that diversity does not correlate with stability in this case, at least by these measures. The recovery of the community as measured by morphotype similarity analysis showed that the high spirochete reactor returned to its original community composition, while the low spirochete reactor did not (Figure 5). The ARDRA analysis confirmed the morphotype analysis, both showing that Spirochetes, which initially dominated the high spirochete reactors, were diluted by

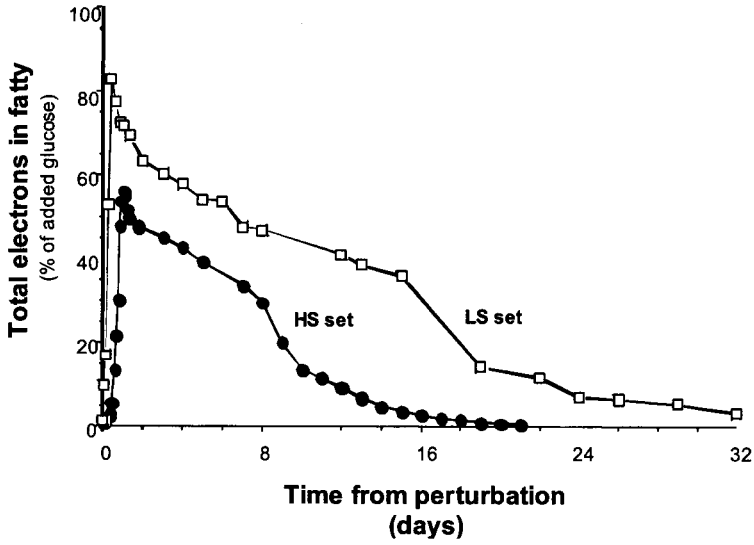

Figure 4. Recovery of community function measured as % of electron flow in fatty acids following the glucose shock-loading at day 0.

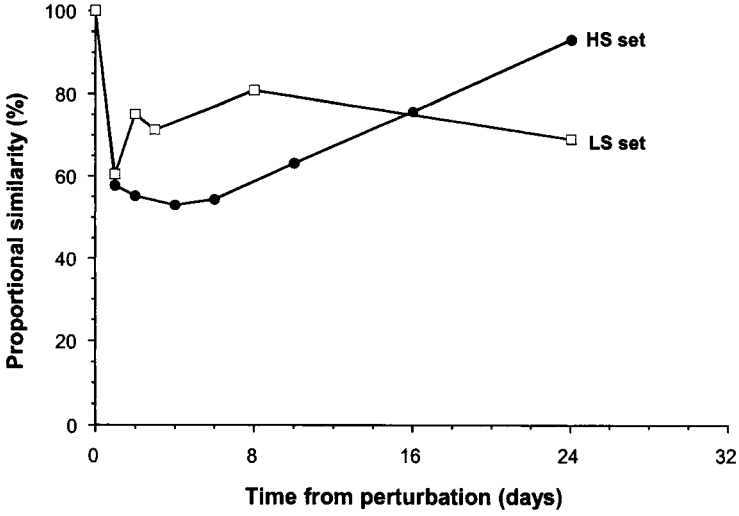

Figure 5. Recovery of the community structure as measured by image analysis following the glucose shock-loading at day 0.

organisms that grew faster on the glucose, but then returned to dominance at day 16 and thereafter following the glucose perturbation.

The high spirochete reactors appeared to accommodate the high glucose loading because minor members of the population could rapidly ferment the extra glucose producing butyrate. ARDRA and 16S rDNA sequence analysis showed that this fast responding population was related to *Eubacterium hadrum*.The large population of Spirochetes in a high spirochete community is thought to ferment glucose to acetate, but when a large amount of glucose was fed, a fast growing *Eubacterium* that was initially present in very low numbers emerged, out competing the slower growing Spirochete populations, eventually shifting 30% of the electron and carbon flow through butyrate (Figure 6). This was accompa nied by a high proportion of fast-growing, acetoclastic *Methanosarcina* species which rapidly metabolized the acetate generated after the perturbation, resulting in little accumulation of acetate.

In the low spirochete reactor, *Streptococcus*-like organisms responded to the glucose addition and produced lactate which appeared to be followed by clostridia converting the lactate to butyrate. Acetate subsequently accumulated because the methanogenic population was dominated by the slow growing *Methanosaeta* species. Organisms like *Eubacterium* and *Clostridium* probably produce large amounts of H_2 shifting the reducing equivalents directly to a neutral methanogenic substrate that is rapidly utilized and possibly conferring functional stability to the system.

Our studies show that functional stability could not be attributed to higher species diversity, rather, a flexible community structure allowed minor members to rapidly accommodate the high flux of glucose. Consequently our conclusion is that a stable community structure may be inflexible and does not allow populations to quickly respond to new conditions. Hence, flexibility rather than diversity or persistence may be important traits for stably functioning reactor ecosystems.

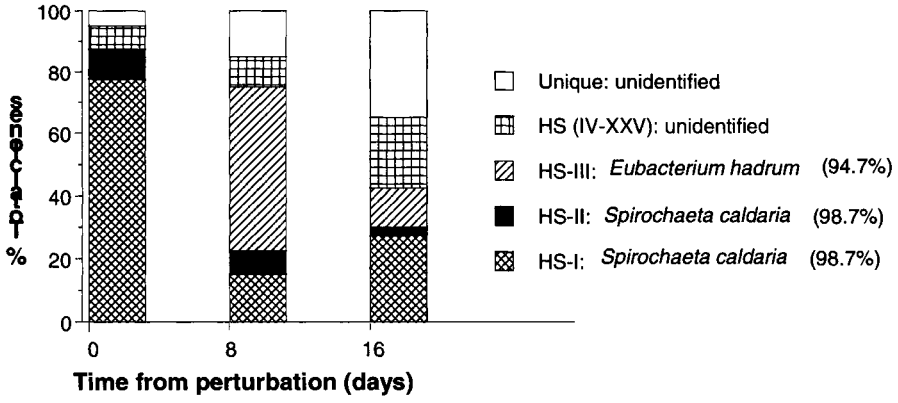

Figure 6. Succession of OTU's in the high spirochete reactor following the glucose shock-loading at day 0.

ACKNOWLEDGMENTS

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REFERENCES

1. Fernandez, A., Hashsham, S.A., Dollhopf, S.L., Raskin, L., Dazzo, F.B., Glagoleva, O., Hickey, R.F., Criddle, C.S., and Tiedje, J.M. (2000). Appl. Environ. Microbiol. (In Press).
2. Fernandez, A., Huang, S., Seston, S., Xing, J., Hickey, R.F., Criddle, C., and Tiedje, J.M. (1999). Appl. Environ. Microbiol., Vol 65, pp3697-3704.
3. Hashsham, S.A., Fernandez, A.S., Dollhopf, S.L., Dazzo, F.B., Hickey, R.F., Tiedje, J.M. and Criddle, C.S. (2000). Appl. Environ. Microbiol. (In Press).
4. Xing, J., Criddle, C., and Hickey, R. (1997). Microb. Ecol. Vol 33, pp50-58.
5. Massol-Deya, A., Odelson, D.A., Hickey, R.F., and Tiedje, J.T. (1995). In: *Molecular microbial ecology manual*, A.D.L. Akkermans, J.D. van Elsas, and F.J. de Bruijn (eds.), Kluwer Academic Publishers, Dordrecht, The Netherlands, p. 3.3.2:1-8.
6. Hashsham, S., Marsh, T., Dollhopf, S., Fernandez, A., Dazzo, F., Hickey, R., Criddle, C., and Tiedje, J. (2000). In this volume.
7. Grimm, V., Schmidt, E., and Wissel, C. (1992). Ecological Modelling, Vol 63, pp143-161.
8. Neubert, M.G. and Caswell, H. (1997). Ecology, Vol 73, No. 3, pp653-665.

Strategic approach for characterization of bacterial community in enhanced biological phosphate removal (EBPR) process

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The present paper focuses on possible strategies to highlight bacterial populations playing key roles in EBPR processes. Three experimental approaches are presented. First, measurement of acetoacetyl-CoA reductase activity implies that the amount of the enzyme actually present is much higher than the amount that is needed to ensure the observed PHB synthesis rate. It is likely that acetoacetyl-CoA reductatse is an intrinsic (not necessarily a rate-limiting) enzyme in the PHA production, thus a key enzyme for EBPR. It is also indicated that the source of reducing power for this enzyme can be different among sludges. Acetoacetyl-CoA reductase should be metabolically (and thus genotypically) diverse. Second, fluorescence *in situ* hybridization is applied to characterize bacterial community of activated sludge. There is no significant difference in the bacterial structure between the non-EBPR activated sludge and the EBPR activated sludge. It is likely that possible differences, if any, are not reflected on the subgroup level bacterial structures. Third, very diverse nature of the EBPR community is demonstrated by the polymerase chain reaction – denaturing gradient gel electrophoresis. In summary, the assumption that PAOs are phylogenetically diverse must be verified by evidence. It is important to develop as many probes for PAOs and to apply them to as many EBPR sludges as possible together with metabolic characterization of the EBPR sludges.

1. INTRODUCTION

The principle of enhanced biological phosphate removal (EBPR) from wastewater was first found in 1970s [1, 2]. The EBPR process is primarily characterized by circulation of activated sludge through anaerobic and aerobic phases, coupled with the introduction of influent wastewater into the anaerobic phase. Such a configuration is called the anaerobic-aerobic activated sludge process. By this configuration, polyphosphate-accumulating organisms (PAOs) are ecologically selected and grow to dominance in the process. High phosphate removal efficiency can be achieved by withdrawing excess sludge with high phosphorus content. The metabolic behaviors of PAOs have been reasonably explained based on experimental results from mixed culture studies. However, neither a single pure culture has been proven to be one of the predominant bacteria in the EBPR process, nor has a microorganism responsible for EBPR ever been isolate [3]. The present paper first reviews the specific metabolisms and the bacterial community of the EBPR process, and then focuses

on possible strategies to highlight bacterial populations playing key roles in EBPR and presents three experimental approaches to obtain a clearer picture of the EBPR community.

2. MINI REVIEW ON EBPR METABOLISM AND BACTERIAL COMMUNITY

2.1. EBPR Metabolism

It was demonstrated [4,5] that the phosphate removed in the EBPR process is stored as polyphosphate (poly-P) in the sludge. PAOs can utilize stored poly-P as energy source for the uptake of carbon sources in the anaerobic phase of EBPR processes [6], whereas aerobic heterotrophs can not get energy for anaerobic substrate uptake. Hence, the anaerobic phase should serve as a selector for PAOs.

Because PAOs thrive on short chain fatty acids (SCFAs) like acetate [7] metabolism of SCFAs, especially acetate, has been extensively studied. Acetate or other SCFAs incorporated into PAOs is stored in the cell in the form of polyhydroxyalkanoates (PHA) accompanied by concomitant degradation of poly-P and consequent release of orthophosphate. In the subsequent aerobic phase, PAOs grow aerobically and take up orthophosphate to recover the poly-P level by using the stored PHA as the carbon and energy source. Since PHA is a reduced polymer, its synthesis requires reducing power. The reducing power is considered to be produced from partial oxidation of intracellularly stored glycogen to carbon dioxide along with production of acetyl-CoA [8-10]. It was also proposed [11] that the glycogen can be metabolized to propionyl-CoA through the succinate-propionate pathway consuming reducing power. In actual EBPR processes, PAOs have to be ready for the uptake of various kinds of reduced or oxidized organic substrates in the anaerobic phase without disturbing the redox balance in the cell. The function of stored glycogen to maintain the redox balance, therefore, appears to be essential for the anaerobic uptake of various organic substrates, and thus for the proliferation of PAOs in the EBPR [3,12].

2.2. EBPR Bacterial Community

In the 1980s, *Acinetobacter spp.* were believed to be responsible for poly-P accumulation and thus for EBPR, because culture-dependent identification of bacteria indicated that dominance of *Acinetobacter* was often observed in activated sludge with high P removal performance [4,13,14]. However, it is now demonstrated based on non-culture-dependent techniques that *Acinetobacter* is not primarily responsible for EBPR. For example, application of 16S-rRNA targeting oligonucleotide probes, which are specific for *Acinetobacter* or other bacterial groups, showed that culture-dependent methods strongly benefited *Acinetobacter* and that *Acinetobacter* was not dominant (less than 10% of total bacteria) in the EBPR processes studied [15-17].

In the past, it may have been believed that an EBPR community should be enriched with a single dominant group of bacteria. Recently, molecular techniques have revealed that the bacterial community of the EBPR process is phylogenetically diverse and consist of several major bacteria [15,16,18,19]. Further, molecular techniques have also shown possible phylogenetic positions of PAOs. For example, double staining of an EBPR sludge with DAPI for poly-P and with oligonucleotide probes for some phylogenetic subclasses demonstrated [20] that the alpha subclass of proteobacteria and the Gram positive bacteria with a high G+C DNA content accumulated poly-P in the EBPR process. This implies that PAOs belong to these two bacterial subgroups. An acetate fed activated sludge with high EBPR capacity

was proven to be dominated (80% of DAPI-stained cells) by *Rhodocyclus* group, which belong to the gamma subclass of proteobacteria [21]. It is strongly implied that PAOs are not a certain bacterium, but composed of a few dominant bacterial groups.

3. ENZYMOLOGICAL NATURE OF EBPR SLUDGES

3.1. Experimental Methods

PHA synthesis is an intrinsic metabolism for PAOs to enable anaerobic substrate uptake. Identification of the key reactions that regulate the whole metabolism should lead to better understanding of the EBPR mechanism and to clearer characterization of PAOs. In order to investigate the importance of various enzymes in the EBPR metabolism, activities of 8 enzymes in and around the PHA synthesis pathways were measured: the enzymes include beta-ketothiolase (KT), NADH-linked acetoacetyl-CoA reductase (AARe), NADPH-linked acetoacetyl-CoA reductase (P-AARe), pyruvate kinase, pyruvate carboxylase, phosphoenolpyruvate carboxylase, malate dehydrogenase (MDH) and lactate dehydrogenase. Enzymes were extracted from activated sludges into 0.1 M tris-HCl buffer (pH 7.5) by sonication (20Watt for 5 minutes) at 4 degrees C, and separated by centrifugation at 13000 rpm for 30 min. The crude extract was used for enzyme activity measurement. In all the enzyme activity measurements, appropriate reaction systems were developed so that the reaction rate could be expressed as the decrease of NADH or NADPH [22,23]. Different sludge samples from 15 activated sludge systems including full- and lab-scale processes or conventional (fully aerobic) and anaerobic-aerobic processes were subjected to the enzyme activity measurement.

3.2. Results

Among the 8 enzymes studied, activities of P-AARe and AARe for reduction of acetoacetyl-CoA to hydroxybutyryl-CoA, KT for dimerization of acetyl-CoA to acetoacetyl-CoA and MDH for conversion of oxalacetate to malate were significantly higher in the sludges from lab-scale anaerobic-aerobic reactors fed with acetate-rich wastewater than in other sludges. In Figure 1, the activities of P-AARe and KT are plotted against the observed maximum PHB synthesis rates in acetate fed aerobic batch experiments using various sludges. It can be seen that the KT activities are almost comparable to the observed PHB synthesis rates, whereas P-AARe activities are much higher (12 times higher in the highest case). This means that the amount of P-AARe actually present is much higher than the amount that is needed to enable the observed maximum PHB synthesis rate. The activities of NADH-linked AARe were higher than that of P- (NADPH-linked) AARe in most of the sludge. In the anaerobic phase of the EBPR process, the NADPH or NADH concentration may be very low due to limited production of reducing power. The strategy which should be usually adopted by microorganisms under low substrate concentration conditions may be to

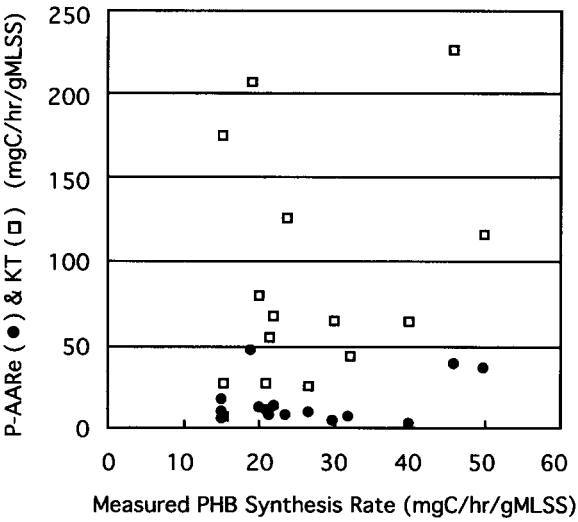

Figure 1. Relationship between PHB synthesis rate of activated sludge and activities of extracted NADPH-linked acetoacetyl-CoA reductase (P-AARe) and ketothiolase (KT).

lower the half saturation constant (Ks) in the Michaelis-Menten equation (in other words, to increase the affinity of the enzyme to the substrate). However, PAOs appear to solve the problem not by lowering Ks for P-AARe or AARe but by increasing the amount of the enzyme, thus increasing the maximum activity of P-AARe or AARe. This is unusual, and the reason is not clear. It is likely that acetoacetyl-CoA reductatse is an intrinsic (not necessarily a rate-limiting) enzyme in the PHA production, thus a key enzyme for EBPR.

Table 2
Activity of NADH- and NADPH-linked acetoacetyl-CoA reductase from lab-scale anaerobic aerobic activated sludge processes

Sludge	Substrate Composition (Ac:Pr:Pe:Ye)	MLSS (mg/L)	Sludge Retention Time (day)	NADPH-linked AARe Activity (unit/mgProtein) [A]	NADH-linked AARe Activity (unit/mgProtein) [B]	Ratio [B]/[A]
(a)	25:35:30:10	800-1000	5	0.53	1.69	3.2
(b)	25:35:30:10	1000-1400	5	0.49	1.50	3.1
(c)	80: 0:12: 8	600-1000	5	0.71	2.12	3.0
(d)	20:60:12: 8	1000-1500	5	0.51	2.35	4.6
(e)	100: 0: 0: 0	2000-2500	10	0.39	2.89	7.4

Note - Ac:acetate, Pr:propionate, Pe:peptone, Ye:yeast extract
- 1 unit is defined as the amount of enzyme which achieves a reaction rate of 1 μ mol/min.
- mg Protein: mg protein extracted in the crude enzyme extract solution

In Table 2, the activities of AARe and P-AARe from five laboratory anaerobic-aerobic reactors are compared. Both the enzymes convert acetoacetyl-CoA to hydroxybutyryl-CoA. This reaction requires reducing power. The former enzyme uses NADH as the reducing power source, whereas the latter uses NADPH. It can be seen from Table 2 that the ratios of the AARe activity to the P-AARe activity vary among the five sludges tested. In the case of sludge (e), the contribution of NADPH is significantly higher than in other sludges. It seems that the source of reducing power can be different among sludges. Acetoacetyl-CoA reductase should be metabolically (and thus genotypically) diverse.

4. COMMUNITY STRUCTURES OF EBPR ACTIVATED SLUDGES HIGHLIGHTED BY FISH

4.1. Experimental Methods

Florescence *in situ* hybridization (FISH) is a molecular method to detect 16S-rRNA sequences specific for certain phylogenetic microbial groups. In this method, fluorescence-labeled short DNA fragments are hybridized to the complementary sequences of the target rRNA. Thus, the target cells can be detected under a fluorescence microscope. We applied the FISH method to compare bacterial community structures of activated sludges from conventional (fully aerobic) activated sludge process for BOD removal only and anaerobic-aerobic process for EBPR [24]. Three activated sludge samples were collected in a sewage treatment plant in Tokyo, one from a conventional (fully aerobic) process (process C), one from an anaerobic-aerobic process for EBPR (process AO) and another from an anaerobic - anoxic - aerobic process for nitrogen and phosphate removal (processA_2O). These three processes treated the same sewage and the possible difference in the bacterial community structure should be dependent on the design and the operation of each process. Six 16S-rRNA targeting oligonucleotide probes were selected; they are probes specific for the alpha, beta and gamma subclasses of proteobacteria (ALF, BET, GAM, respectively), Gram positive bacteria with a high G+C DNA content (HGC), Cytophaga-Flavobacterium (CF) and eubacteria (EUB) [25]. Hybridization was performed according to Amann (1995) [25]. The fluorescent area was measured by a confocal laser scanning microscope (Leica, TCS-NT) and the ratio of each bacterial subgroup was expressed as percentage against EUB.

4.2. Results

The results of four surveys (Autumn 1997 – Summer 1998) are summarized in Figure 2. There is no significant difference in the bacterial structure between the non-EBPR activated sludge (C) and the EBPR activated sludge (AO and A_2O). It is likely that possible differences, if any, are not reflected on the subgroup level bacterial structures. As PAOs may belong to various phylogenetic groups [20,21], this implication is understandable.

5. MONITORING EBPR COMMUNITY BY PCR-DGGE METHOD

5.1. Experimental Methods

Among molecular techniques, the polymerase chain reaction (PCR) - denaturing gradient gel electrophoresis (DGGE) method is a powerful tool to analyze undefined microbial communities. In this method, DNA is extracted from biomass samples and a certain target

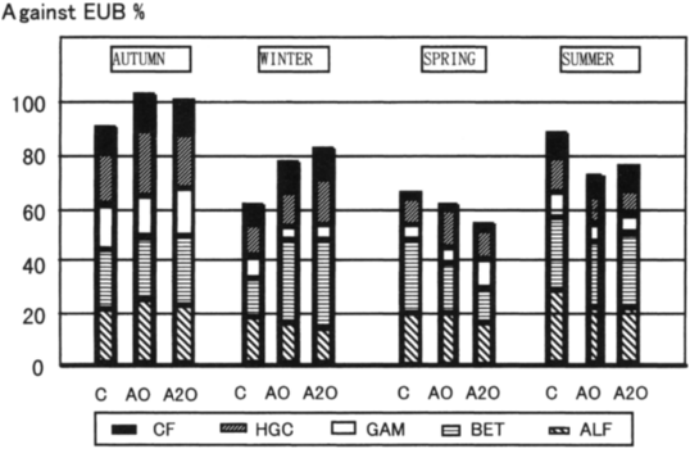

Figure 2. Comparison of bacterial community structures of activated sludges from conventional (C), anaerobic-aerobic (AO) and anaerobic-anoxic-aerobic processes (A_2O).

region of the DNA defined by a primer set is amplified by PCR. The amplified DNA fragments with different sequences can be physically separated based on the difference in the DNA sequences. In general, DNA fragments with higher G+C DNA contents tend to be denatured less easily, thus migrate more in the electrophoresis gel. Each DNA band on the gel corresponds to a DNA fragment with a certain sequence. The complexity of the band pattern indicates the diversity of the DNA sequence, thus the biological diversity in general. We applied the PCR-DGGE method to monitor an EBPR community in a lab-scale anaerobic-aerobic process operated with a complex synthetic wastewater. DNA was extracted from sludge samples by using the benzyl chloride method [26]. PCR amplification with the primers for the highly variable V3 region (341f-GC and 534r) [27] was performed using Ampli Taq Gold DNA polymerase (Perkin-Elmer Japan, Co Ltd.) and Gene Amp PCR System Model 9600 (Perkin-Elmer). The amplified DNA was subjected to DGGE according to Onuki *et al.* (1999) [28].

5.2. Results

Examples of the EBPR bacterial community monitored by DGGE are shown in Figure 3. Samples A & B and C & D were obtained on February 8 & 12, and May 6 & 14, respectively, in 1998. The band patterns of A and B are similar to each other; those of C and D, too. It appears that bacterial community does not change much within one week or so. However, there was a significant change in bacterial community between February and March, although both sludges looked similar in terms of EBPR performance. There are many visible bands which implies complex and diverse nature of the EBPR sludge. For further phylogenetic characterization, it is necessary to obtain sequence information of these bands.

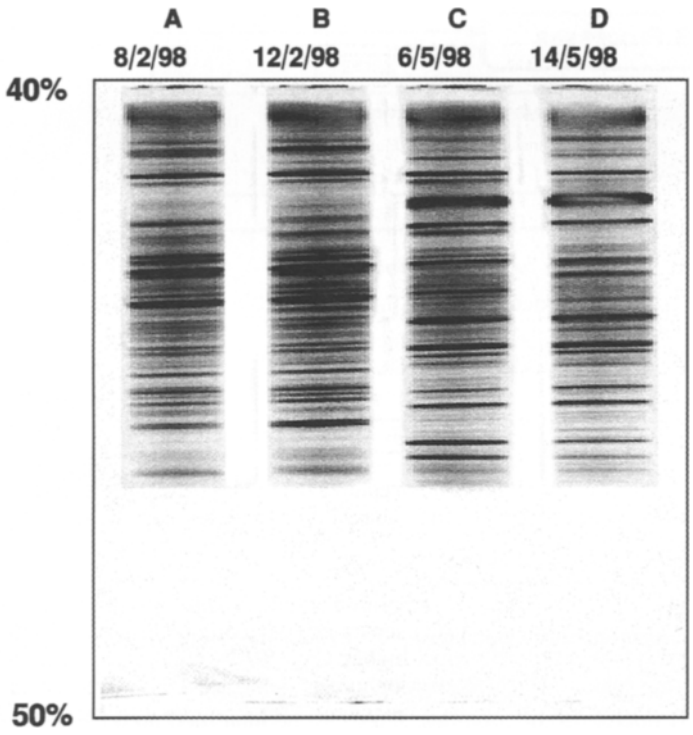

Figure 3. Bacterial Community of an EBPR Sludge Monitored by PCR-DGGE Method

6. PROPOSED STRATEGIC APPROACH

Metabolic diversity of PAOs is implied through the measurement of enzyme activities for PHA synthesis. Phylogenetic diversity is also indicated through the application of the FISH and PCR-DGGE methods, as has been shown in many other studies. It is likely that diverse organisms are responsible for EBPR. The principal PAOs in an EBPR system may vary from case to case. The assumption that PAOs are phylogenetically diverse and that different EBPR systems can be dominated by different PAOs, must be verified by evidence. For this purpose, bacterial community structures of as many different EBPR systems with the typical EBPR metabolic characteristics should be investigated.

We propose a strategic approach to characterize the EBPR microbial community and to understand the microbiological mechanism of the EBPR process more clearly. The approach is schematically shown in Figure 4. This approach consists of two major concepts: metabolic characterization of PAOs and *in situ* quantification of PAOs. By using molecular approaches like the PCR-DGGE method, cloning, etc., DNA sequences of PAO candidates

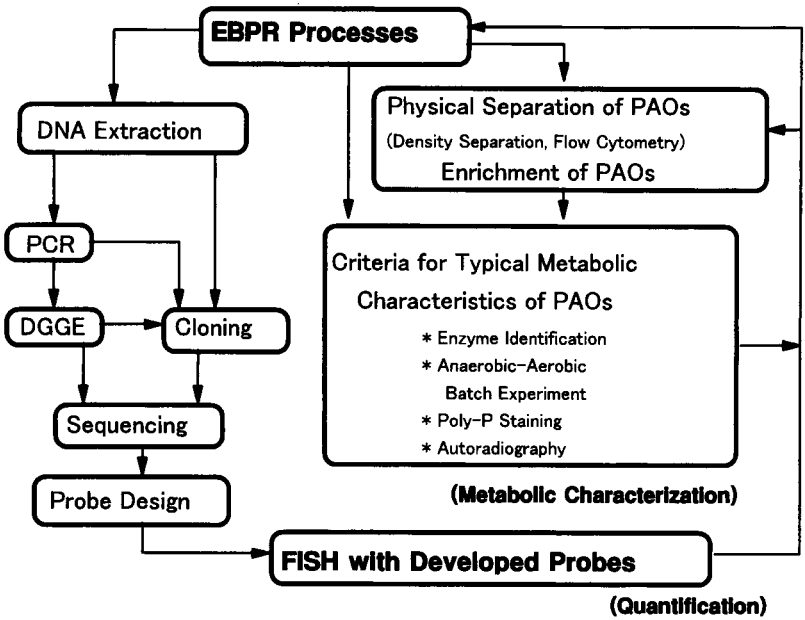

Figure 4 Proposed Strategic Approach for Characterization of EBPR Community

can be obtained from EBPR microbial communities. They will be identified on a molecular level. This leads to probe design targeting these molecular isolates. With these probes developed, *in situ* quantification of the target bacteria will become possible and we can investigate who are dominant in various EBPR processes. However, these molecular isolates should not be recognized as PAO without verifying that they really exhibit typical metabolic characteristics of PAOs. If we can establish a phylogenetically simple EBPR community that preferably contains very limited number of major molecular isolates, then morphological observation like poly-P staining may be able to indicate that the obtained molecular isolates possess the PAO's metabolic characteristics. Alternatively, double staining for poly-P and 16S-rRNA[20] can be a promising measure to find PAOs *in situ*. More sophisticated simultaneous application of FISH and microautoradiography [29] must be also very promising to see if the target bacterium really accumulate poly-P in the anaerobic phase.

7. CONCLUSIONS

It is important to develop as many probes for PAOs and to apply them to as many EBPR sludges as possible along with metabolic characterization of the EBPR sludges tested. Without investigating a wide range of EBPR sludges, an overall view on the EBPR microbial community may not be clarified. Simultaneous application of 1) detection or quantification of microorganisms in well-defined EBPR processes and 2) confirmation from metabolic

point of view that the detected or quantified organisms surely do the EBPR performance, is essential.

REFERENCES

1. Barnard J.L., Water Research, 9 (1975) 485.
2. Fuhs G.W. and Chen M., Microbial. Ecol., 2, (1975) 119.
3. Mino T., van Loosdrecht M.C.M. and Heijnen J.J., Water Res., 32, (1998) 3193.
4. Buchan L.,Wat. Sci. Tech., 15 (1983) 87.
5. Mino T., T. Kawakami and T. Matsuo, Wat. Sci. Tech., 17, Amsterdam (1984) 93.
6. Mino T., T. Kawakami and T. Matsuo, Wat. Sci. Tech., 17, Paris (1985) 11.
7. Wentzel M.C., Lotter L.H., Loewenthal R.E. and Marais G.v.R. Water SA, 12, 4 (1986) 209.
8. Mino T. and Matsuo T., Japanese J. Water Pollut. Res., 7 (1984) 605 (in Japanese).
9. Mino T., Tsuzuki Y. and Matsuo, T., Adv. Wat. Pollut. Cont., Biological Phosphate Removal from Wastewaters, Pergamon Press (1987).
10. Arun V., Mino T. and Matsuo T. Water Research, 22, (1988) 565.
11. Satoh H., Mino T. and Matsuo T., Wat. Sci. Tech., 26 (1992) 933.
12. Mino T., Satoh H. and Matsuo T., Wat. Sci. Tech., 29 (1994) 67.
13. Lotter L.H., Wat. Sci. Tech., 17, (1985) 127.
14. Wentzel M.C., Loewenthal R.E., Ekama G.A. and Marais G.v.R. (1988) Water SA, 14, 2 (1988) 81.
15. Wagner M., Erhart R., Manz W., Amann R., Lemmer H., Wedi D. and Schleifer K.-H., Appl. Environ. Microbiol., 60 (1994) 792.
16. Kampfer P., Erhart R., Beimfohr C., Bohringer J., Wagner M. and Amann R., Microb. Ecol., 32 (1996) 101.
17. I Made Sudiana, Satoh H., Mino T. and Matsuo T., Wat. Sci. Tech., 38 (1998) 69.
18. Bond P., Hugenholtz P, Keller J. and Blackall L., Appl. Environ. Microbial., 61 (1995) 1910.
19. Liu W.T., Marsh T. and Forney J.L., Wat. Sci. Tech., 37, 4-5 (1998) 417.
20. Kawaharasaki M., N. Tanaka, T. Kanagawa and K. Nakamura,Water Res., 33 (1998) 257.
21. Hesselmann, R.P.X., C. Werlen, D. Hahn, J.R. van der Meer and A. J. B. Zehnder, System. and Appl. Microbiol., accepted (2000).
22. Nishimura, T., Fukui, T. and Ikeda K., Arch. Microbiol. 116 (1978) 21.
23. Saito, T., Fukui, T. and Ikeda F., Arch. Microbiol. 114 (1977) 211.
24. Akiyama T., Satoh H., Mino T. and Matsuo T., J. Japan Soc. Water Environ., 23 (2000) in press (in Japanese).
25. Amann, R.I., Molecular Ecological Manual, Kluwer Academic Publisher, Dordorecht, the Netherland (1995) 3.3.6/1.
26. Zhu H., Qu, F., and Zhu, L-H., Nucleic Acids Res. 21 (1993) 5279.
27. Muyzer, G., Waal, E.C. and Uitterlinden A.G., Appl.. Environ. Microbiol. 59 (1999) 695.
28. M. Onuki, H. Satoh, T. Mino and T. Matsuo, Proc. Asian IAWQ Regional Conf., Taipei, ROC (1999).
29. Lee, N., Nielsen, P.H., Andreason, K.H., Juretschko, S., Nielsen, J.L., Schleifer, K-H. and Wagner, M., Appl.. Environ. Microbiol. 65 (1999) 1289.

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Microbial community structure and their activity in aquatic environment

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Microbes process and degrade various compounds in the environment, which means they have profound potentials to directly purify the polluted environments. In order to apply their abilities appropriately, we need to understand what kind of microbes exist, where and how many there are, how they live, and what interactions they perform among themselves and with environment. The recent advances in methodology lead us to the new era of microbiology. They allow us to understand microbes in aquatic environment from quite accurate quantitative point of view. Based on the knowledge of microbial ecology accumulated so far in temperate zones, novel findings on microbes in tropical zones should further enable us to understand microbial ecology on a global scale. Here, we applied new techniques to analyze microbial community structure in tropical river waters.

1. FLUORESCENT VITAL STAINING AND FLUORESCENT IN SITU HYBRIDIZATION

Certain fluorescent dyes are available for the detection of physiologically active bacterial cells by epifluorescence microscopy and flow cytometry. 6-carboxyfluorescein diacetate (6CFDA), which is hydrolyzed to green-fluorescent 6-carboxyfluorescein by non-specific esterases inside the cell, is useful in examining bacterial esterase activity [1-4]. 5-cyano-2,3-ditolyl tetrazolium chloride (CTC), a tetrazolium salt, which is reduced by respiring bacteria to a water-insoluble, red-fluorescent CTC-formazan, has been employed as an indicator of bacterial respiratory function [3-9].

Fluorescent in situ hybridization (FISH) using rRNA-targeted probes has been used to detect and identify bacteria in natural aquatic environment [10-12]. 2-hydroxy-3-naphthoic acid-2'-phenylanilide phosphate (HNPP) and Fast Red TR in situ hybridization (HNPP-FISH) [13], which enhances fluorescent signals eightfold compared to standard FISH with

mono-FITC-labeled oligonucleotide probes, can be used to analyze bacterial community in oligotrophic aquatic environment [14]. We used 6CFDA staining [3] for enumeration of esterase-active bacterial cells and also HNPP-FISH technique [14] for analysis of bacterial community structure in natural rivers.

Surface river water was collected at four sites (MN1, MN2, IN3 and NY4) in the northern part of Osaka, Japan (Figure 1), on March 7, 1998. MN1 and MN2 on Minoh River are located in a mountain area. MN1 is located upstream of MN2 and its environment is similar to MN2. IN3 on Ina River is located in an industrial area. NY4 on Neya River is located in a commercial area, Osaka Business Park, and domestic water flows into this river upstream. Surface river water was also collected at five sites (KL1, KL2, KL3, KL4 and SA5) on Kelang River basin, Malaysia (Figure 1) on November 29, 1997. KL1, KL2, KL3 and KL4 are located in Kuala Lumpur, and domestic water flows into these sites. SA5 is located in an industrial area of Shah Alam.

Table 1 shows physico-chemical water quality of the nine river water. Ambient temperature and river water temperature on the sampling date in Japan were lower than 15°C, while those in Malaysia were higher than 25°C. Total organic carbon (TOC) concentration indicates that MN1 and MN2 are oligotrophic sites and other sampling points are eutrophic.

The number of colony forming units (CFU) determined by R2A medium [15], number of esterase active bacteria and total bacterial number are shown in Figure 2. Total bacterial number was determined by 4',6-diamidino-2-phenylindole (DAPI)-staining. Estimates of culturable bacteria on R2A medium accounted for 0.5 - 3 % of DAPI-stained bacteria at MN1, MN2 and IN3 of river in Osaka, Japan, 13 % at NY4 of Osaka, Japan and 12 - 31% of Kuala Lumpur, Malaysia. The ratios of CFU to total bacterial number are quite different between

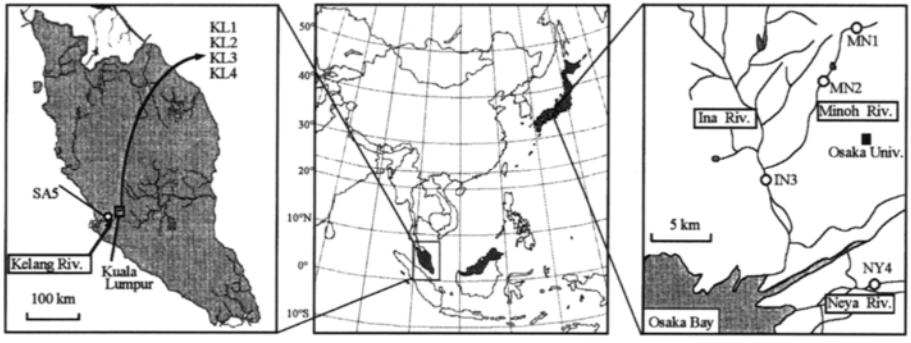

Figure 1. Sampling stations

Table 1
Physico-chemical characteristics of river water

Property	MN1	MN2	IN3	NY4	KL1	KL2	KL3	KL4	SA5
Ambient temp. (ÞC)	8.3	8.2	13.0	14.6	25.7	27.5	26.2	29.0	32.6
Water temp. (ÞC)	8.0	6.0	11.0	16.8	26.9	27.4	26.0	27.8	28.6
pH	7.9	7.5	7.5	7.2	7.7	7.6	7.4	7.5	7.1
Total organic carbon (mg Þ^{1})	1.0	0.8	4.4	10	6.0	5.4	4.0	5.5	13

IN3 (2.5 %) and samples of Kelang River basin (12 -31 %) , while total bacterial number and TOC values of these samples were similar (Figure 2, Table 1). The numbers of esterase active bacteria (6CFDA(+)) were similar to CFU in the Malaysian river water samples, while 6CFDA(+) were five to 100 times higher than CFU in water samples taken from rivers in Osaka. These data suggest that bacterial activity in tropical rivers should be different from that in temperate rivers, while optimal medium and staining condition of 6CFDA for bacterial cells in tropical river water samples may be examined in detail.

Bacterial community structure of seven river water samples were determined by HNPP-FISH (Figure 3). The following oligonucleotides were used [14]: i) *Flavobacterium* - *Cytophaga*

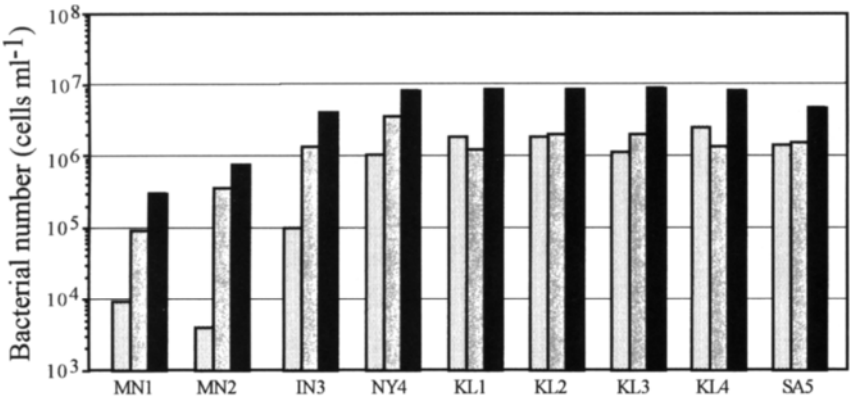

Figure 2 . Bacterial number in river water samples taken from Minoh River, Ina River, Neya River and Kelang River basin. □, colony forming units on R2A medium. ▨, number of esterase-active bacteria determined by 6CFDA-staining. ■, total bacterial number determined by DAPI-staining.

(5'-AGGTACCCCCAGCTTCCATGGCT-3'), ii) *Burkholderia-Rubrivivax-Bordetella* (5'-GTGTGCCGGTTCTCTTTCGAGCAC-3'), iii) *Acinetobactor* (5'-GCGCCACTAAAGCCTCAAAGGCC-3'), iv) *Vibrio-Aeromonas* (5'-ACGACGCACTTTTTGGGATTCGCTCACTATCGCAAG-3'), v) *Pseudomonas* I (5'-ATTTCAGCCTACCACCTTAA-3'), and vi) EUB338 (5'-GCTGCCTCCCGTAGGAGT-3') for domain Bacteria [16]. Forty to 70 % of total cells could be detected with EUB338 probe in the river water samples used in this study. The bacterial community in river water samples taken from Minoh River and Neya River in Japan was dominated by *Flavobacterium-Cytophaga* group (10 % at MN1 and 30% at NY4) and *Burkholderia-Rubrivivax-Bordetella* group (10 % at MN1 and 13 % at NY4). Predominance of *Flavobacterium* in temperate rivers and lakes determined by cultivation-based methods has been reported [17-22] and our results are consistent with those reports. The *Acinetobacter* group dominated (21 - 38 % of total bacterial cells) in the samples from Kelang River basin, Malaysia. Bacterial community structure was quite different between temperate river samples and tropical river samples.

In this study, we determined the bacterial enzymatic activity and bacterial community structure in natural river water in Osaka, Japan and Kuala Lumpur, Malaysia. Twenty to 50 % of total bacterial cells had physiological activity, and bacterial community structure was dominated by *Flavobacterium-Cytophaga* group in temperate rivers and by *Acinetobacter* group in tropical rivers. Further analysis of the physiology of members of the predominant genera will increase our understanding of their roles in nature, and studies combining modern

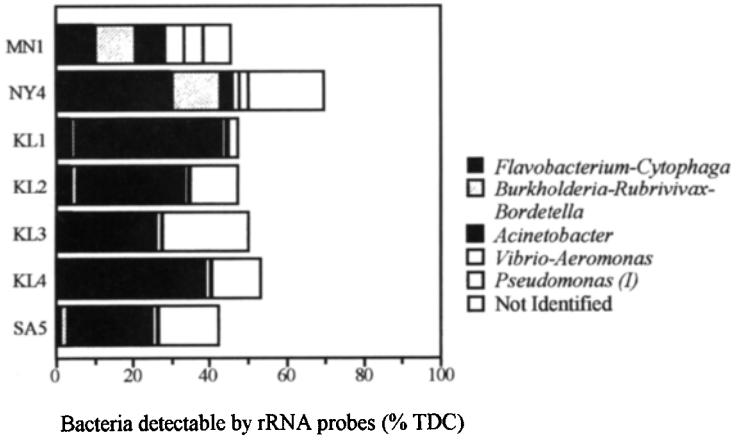

Figure 3. Comparison of bacterial community structure in river water samples taken from Minoh River, Neya River and Kelang River basin

molecular and traditional culturing-based techniques should give new insights into the ecology of rivers.

Bacterial cells with low metabolic activity can be identified by in situ PCR [23], and could also provide information about specific genes and their transcripts at a single cell level [24,25]. Bacterial community structure in these river water samples can be analyzed from the functional point of view by this in situ PCR technique.

2. DENATURING GRADIENT GEL ELECTROPHORESIS (DGGE)

DGGE can separate DNA fragments whose length are the same but sequences are different [26-33]. Since its initial application to environmental 16S ribosomal DNA, this molecular technique has been used extensively in microbial ecology. The number of bands observed in DGGE profiles provides an estimate of species richness. The relative intensity of each band provides a rough estimate of relative abundance of each species. The bacterial community structure of river water samples was analyzed by this DGGE technique [34].

Figure 4 shows the DGGE profiles of 16S rDNA fragments targeting domain Bacteria obtained from river water. Natural river water was taken at KL1, KL3, KL4 (Kelang

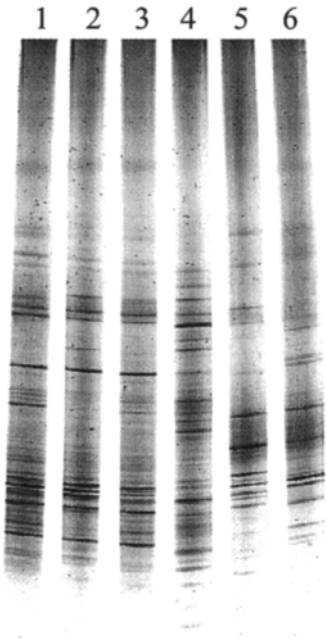

Figure 4. DGGE analysis of PCR-amplified 16S rDNA fragments from river water.
lane 1, KL1;
lane 2, KL3;
lane 3, KL4;
lane 4, NY4;
lane 5, MN2 in February,
lane 6, MN2 in May.

River in Kuala Lumpur, Malaysia; Figure 1), NY4 (Neya River in Osaka, Japan; Figure 1) and MN2 (Minoh River in Osaka, Japan; Figure 1). 16S rDNA fragments (position 933-1387) were amplified with universal primers for bacterial rDNA sequence. A 40 base GC-rich sequence was attached to the 5' end of one primer. The banding pattern of lane 1 is similar to lane 2 and 3, but not to lane 4, 5 or 6. The pattern of lane 5 is similar to lane 6 but different from the others.

Quantitative pattern analysis is essential to clarify the differences of bacterial community structure between one sample and the other, which is indicated by the DGGE profiles, thus metric multidimensional scaling (MDS) [35] was applied. MDS is a mathematical technique which generates a spatial configuration map of data points where distances between points reflect the relationships of individual data. MDS was applied to the DGGE banding pattern analysis to illustrate the similarity of all possible pairs of each gel track. DGGE gel stained with SYBR Gold was scanned with FluorImager (Molecular Dynamics) and digital image was analyzed by Image QuaNT (ver. 4-2-J) to generate a densitometric profile. Similarity of the banding pattern of each gel truck was calculated, based on the position and probability of the band and analyzed by MDS analysis using SPSS 9.0J for Windows (SPSS Japan Inc.) at Genome Information Research Center, Osaka University.

Figure 5 is a two-dimensional plot of MDS score. The positive and negative values placed along the X and Y axis are simply for plotting purpose. By visual inspection of the configurations, the banding patterns among Malaysian samples looked similar and those among Minoh River samples appeared similar, but the pattern of the sample from Neya River

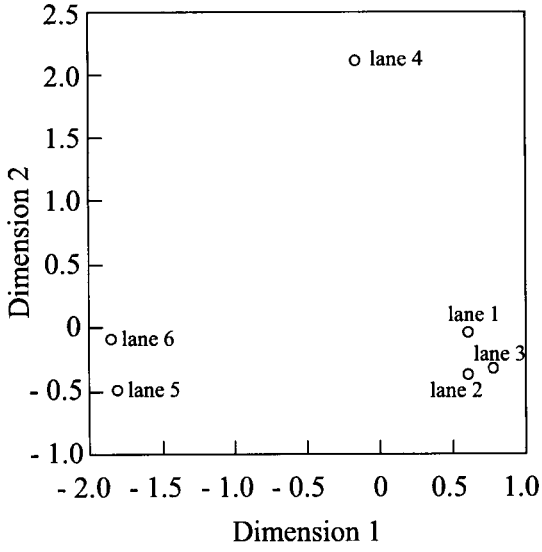

Figure 5. Two-dimensional plot of multidimensional scaling stimulus scores for DGGE banding pattern from river water samples.
lane 1, KL1;
lane 2, KL3;
lane 3, KL4;
lane 4, NY4;
lane 5, MN2 in February,
lane 6, MN2 in May.

seemed different from the others. Since environmental samples usually show highly complex DGGE banding patterns, a quantitative pattern analysis makes the DGGE method a more powerful tool for analyzing microbial community structures. We have to note, however, that the number and intensity of bands do not correspond to the number and abundance of species actually present in the bacterial community, due to possible unknown PCR bias and differences in gene copy number between species. Also, DGGE does not reflect minor species in the environments. Despite these limitations, molecular methods can reveal the presence of microorganisms that can not be detected by classical cultivation techniques.

In this study, we analyzed bacterial community structure in temperate rivers and tropical rivers by DGGE. The results indicate their community structure, which was determined by DNA extracted from total bacterial cells in the samples, should be quite different between temperate rivers and tropical rivers, and they agreed with the results of community analysis determined by fluorescent in situ hybridization at a single cell level.

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REFERENCES

1. C. Dive, H. Cox, J. V. Watson and P. Workman. Mol. Cell. Probes. 2 (1988) 131.
2. J.Porter, C. Edwards and R. W. Pickup. J. Appl. Bacteriol. 79 (1995) 399.
3. N. Yamaguchi, T. Kenzaka and M. Nasu. Microb. Environ. 12 (1997) 1.
4. N. Yamaguchi and M. Nasu. J. Appl. Microbiol. 83 (1997) 43.
5. G. G. Rodriguez, D. Phipps, K. Ishiguro and H. F. Ridgway. Appl. Environ. Microbiol. 58 (1992) 1801.
6. A. S. Kaprelyants and D. B. Kell. J. Microbiol. Methods. 17 (1993) 115.
7. J. Coallier, M. Prevost and A. Rompre. Can. J. Microbiol. 40 (1994) 830.
8. F. P. Yu and G. A. Mcfeters. J. Microbiol. Methods. 20 (1994) 1.
9. R. Lopez-Amoros, D. J. Mason and D. Lloyd. J. Microbiol. Methods. 22 (1995) 165.
10. A. Alfreider, J. Pernthaler, R. Amann, B. Sattler, F. O. Glockner, A. Wille and R. Psenner. Appl. Environ. Microbiol. 62 (1996) 2138.

11. F. O. Glockner, R. Amann, A. Alfreider, J. Pernthalar, R. Psenner, K. Trebesius and K. G. Field. Syst. Appl. Microbiol. 19 (1996) 403.
12. J. Pernthaler, F. O. Glockner, S. Unterholzner, A. Alfreider, R. Psenner and R. Amann. Appl. Environ. Microbiol. 64 (1998) 4299.
13. N. Yamaguchi, S. Inaoka, K. Tani, T. Kenzaka and M. Nasu. Appl. Environ. Microbiol. 62 (1996) 275.
14. T. Kenzaka, N. Yamaguchi, K. Tani and M. Nasu. Microbiol. 144 (1998) 2085.
15. D. J. Reasoner and E. E. Gerldreich. Appl. Environ. Microbiol. 49 (1985) 1.
16. R. I. Amann, B. J. Binder, R. J. Olson, S. W. Chisholm, R. Devereux and D. A. Stahl. Appl. Environ. Microbiol. 56 (1990) 1919.
17. B. Lighthart. Can. J. Microbiolol. 21 (1975) 392.
18. S. Maeda. Jpn. J. Limnol. 41 (1980) 163.
19. M. Stopinski. Acta Microbiol. Pol. 30 (1981) 283.
20. D. Nuttall. J. Appl. Bacteriol. 53 (1982) 49.
21. H. Sugita, H. Tanaami, T. Kobayashi and Y. Deguchi. Jpn. J. Limnol. 43 (1982) 27.
22. K. P. Flint. J. Appl. Bacteriol. 59 (1985) 561.
23. R. E. Hodoson, W. A. Dustman, R. P. Grag and M. A. Moran. Appl. Environ. Microbiol. 61 (1995) 4074.
24. K. Kurokawa, K. Tani and M. Nasu. Jpn. J. Bacteriol. 25 (1997) 415.
25. K. Kurokawa, K. Tani, M. Ogawa and M. Nasu. Lett. Appl. Microbiol. 28 (1999) 405.
26. G. Muyzer, E. C. de Waal and A. G. Uitterlinden. Appl. Environ. Microbiol. 59 (1993) 695.
27. M. J. Ferris, G. Muyzer and D. M. Ward. Appl. Environ. Microbiol. 62 (1996) 340.
28. M. J. Ferris, S. C. Nold, N. P. Revsbech and D. M. Ward. Appl. Environ. Microbiol. 63 (1997) 1367.
29. A. E. Murray, C. M. Preston, R. Massana, L. T. Taylor, A. Blakis, K. wu and E. F. DeLong. Appl. Environ. Microbiol. 64 (1998) 2585.
30. A. S. Rosado, G. F. Duarte, L. Seldin and J. D. van Elsas. Appl. Environ. Microbiol. 64 (1998) 2770.
31. T. Brinkhoff, C. M. Santegoeds, K. Sahm, J. Kuever and G. Muyzer. Appl. Environ. Microbiol. 64 (1998) 4650.
32. M. A. Bruns, J. R. Stephen, G. A. Kowalchuk, J. I. Prosser and E. A. Paul. Appl. Environ. Microbiol. 65 (1999) 2994.
33. S. M. Sievert, T. Brinkhoff, G. Muyzer, W. Ziebis and J. Kuever. Appl. Environ. Microbiol. 65 (1999) 3834.
34. T. Iwamoto, K. Tani, K. Nakamura, Y. Suzuki, M. Kitagawa, M. Eguchi and M.Nasu.

FEMS Microbiol. Ecol. 32 (2000) 129.

35. A. Stewward, K. Prandy and R. Blackburn. Nature 245 (1973) 415.

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Analysis of complex microbial community in soil and wastewater treatment processes by cloning method

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1.CLONE ANALYSIS OF SOIL ARCHAEA

1.1 Backgound about soil archaea

The cultivation of soil archaea such as methanogens is difficult and time consuming which greatly hinders the analysis of soil archaeal flora. However, the use of 16S ribosomal DNA (16S rDNA) or ribosomal RNA (rRNA) overcomes this difficulty as it can be carried out without the need for cultivation. These methods have proved to be a powerful tool for the analysis of soil archaeal flora. The method of 16S rDNA sequencing was used by Oyaizu to analyze the methanogenic flora of sludge from a methane-fermenting plant [1]. This paper reports the use of this method for the archaeal flora analyses of paddy soils in Japan.

It is well known that there are many uncultured bacteria in the environments. In recent years phylogenetic positions of the uncultured bacteria have been identified by 16S rDNA sequence analyses [2-5]. Archaeal uncultured clones were detected in marine environments [5,6] and hot springs [7,8]. A crenarchaeotal lineage, which was separated very deeply from culturable crenarchaeota, was found to be predominant in marine environments [5]. Euryarchaeotal clones were also detected in marine environments[6] and hot springs [7,8]. In the course of study about paddy soil archaea we found many unusual archaeal 16S rDNA clones. This paper reports the existence of extremely unusual uncultured archaea in paddy soils.

1.2.Materials and methods for soil archaeal flora analysis

1.2.1.Archaeal and bacterial strains

The microbial strains used were *Methanobacterium wolfei* DSM 2970^T, *Methanobacterium thermoformicicum* strain FORI, *Methanobrevibacter smithii* DSM 861^T, *Methanobrevibacter arboriphilicus* DSM 1125^T and SA (=DSM 7056), *Methanosaeta*

thermoacetophila DSM 4774^T, *Methanosarcina* mazei strain TMA, *Eschrichia coli* K12, *Aeromonas hydrophila* IAM
12337, *Bacillus licheniformis* IAM 13417, *Citrobacter freundii* IAM 12471, and *Clostridium butyricum* IAM 19240. M. *arboriphilicus* SA and *M. mazei* strain TMA were isolated from paddy soil in Kumamoto, Japan by Asakawa *et al.*. [9,10]. Cells of *M. wolfei* DSM 2970, *M. thermoformicicum* FORI, and *M. thermoacetophila* DSM 4774 were supplied by Drs. Y. Kamagata and K. Nakamura, National Institute of Bioscience and Human-Technology, Tsukuba, Japan, *M. smithii* DSM 861 and *M. arboriphilicus* DSM 1125 from Dr. G. Endo, Tohoku Gakuin University, Tagajyoshi, Miyagi, Japan, and *M. arboriphilicus* SA and *M. mazei* TMA from Dr. S. Asakawa, Kyushu National Agricultural Experiment Station, Nishigoshi, Kumamoto, Japan.

1.2.2.DNA extraction from Methanogens and bacteria

The DNA of methanogens and other bacteria was extracted with benzylchloride as described by Zhu *et al.*. [11]. This was cleaned using phenol extraction followed by RNase treatment. DNA yields were measured using a spectrophotometer.

1.2.3.Design of primers

The primers were designed to amplify the region of DNA between position 1100 and 1400 of the 16S rRNA of archaea, based on sequences of methanogen 16S rRNA from the DNA data base. The sequences used in primer design were those of genus *Methanobacterium* (X68717, X71838, M59124, M36508, and Z37156), *Methanococcoides* (X65537), *Methanococcus* (M59125, M59126, M59128, M36507, and M59290), *Methanocorpusculum* (M59147), *Methanoculleus* (M59129 and M59134) *Methanogenium* (M59130 and M59131), *Methanohalophilus mahii* (M59133), *Methanolobus* (M59135), *Methanopyrus* (M59932), *Methanosaeta* (M59146), *Methanosarcina* (M59137, M59138, M59140, and M59144), *Methanosphaera* (M59139), *Methanospirillum* (M60880), and *Methanothermus* (M59145). From these data it is revealed that the 16S rRNA of methanogens does not contain Eco RI and Sal I restriction enzyme sites. A Sal I restriction enzyme site was added to the 5' end of the annealing region of the 1100F primer, and an Eco RI site was added to the same region of the 1400R primer. The following two primers were designed: 1100F, 5'AACCGTCGACAGTCAGGYAACGAGCGAG3'; and 1400R, 5'CGGCGAATTCGT-GCAAGGAGCAGGGAC3'. The annealing region of each primer contained more than 3 nucleotide missmatches to the 16S rDNA of bacteria and eucarya.

1.2.4.Soils

The soils used for archaeal flora analysis were collected from the following paddy fields: Yamaguchi University (Gray Lowland soil), Kagoshima University (Gray Lowland soil), Saitama Agricultural Research Station (Gray Lowland soil), Yamagata-city (Gray Lowland soil), and the University of Tokyo Yayoi plot (Ando soil). In Yamaguchi University the soil samples were collected from three fields designated numbers 1, 2 and 3. The Yamaguchi field No.2 was not fertilized. The Yamaguchi fields 1 and 3 were fertilized with

chemical fertilizer at the rate of 10.9 kg of N, 13.7 kg of P_2O_5, and 13.9 kg of K_2O per 10 an every year. An additional 50 kg/a of rice straw compost was applied to field No. 1. The field of Kagoshima University was not fertilized. At the Saitama Agricultural Experiment Station soils were collected from three fields designated No. 1,2 and 3. The Saitama field No.1 was not fertilized. The Saitama field No. 2 and 3 were fertilized with chemical fertilizer at the rate of 5 kg of N, 8 kg of P_2O_5, and 3 kg of K_2O per 10 a every year and 100 kg/a of wheat compost was applied to field No. 3. In Yamagata-city a soil sample was collected from a field which was managed by a local farmer however the composition of the fertilizer applied to this field is unknown. The plot from which soil samples were taken at the University of Tokyo Yayoi (Ando soil) was not fertilized.

1.2.5.DNA extraction from soil

DNA was extracted from 3 g of wet soil pellet by the method described by Zhu *et al.* [11]. The crude DNA was purified with QIAGEN-tip 20 (QIAGEN Inc. Germany). The purified DNA was electrophoresed on a 1% agarose gel, and the DNA fragments larger than 10 k bp were extracted from the gel.

1.2.6.PCR conditions

16S rDNAs were amplified using the polymerase chain reaction (PCR) and the primers discussed above. The PCR reaction mixture contained 1 unit of Taq polymerase, 50 mM Tris HCl (pH 9.0), 50 mM NaCl, 10 mM MgCl, 200 mM each of dATP, dGTP, dCTP, and dTTP, 20 pmol of each primer and 50 to 100 ng of template DNA in a total volume of 50 ml. Each reaction mixture was heated at 94 °C for 90 sec followed by 35 cycles of 94 °C for 30 sec, 55 °C for 30 sec and 72 °C for 90 sec. This was followed by 72 °C for 180 sec.

1.2.7.Flora analysis of archaea in paddy field.

Flora analysis was carried out using the method described by Oyaizu [1]. Amplified 16S rDNA was digested with Eco RI and Sal I. The 16S rDNA fragments of approximately 300 base pairs were purified by agarose gel electrophoresis and subcloned into the Bluescript SK+ phagemid vector.

For each soil sample approximately 50 colonies were picked up and ten to thirty clones selected for sequence determination between the 1112 and 1379 positions according to the *Escherichia coli* numbering system. Sequencing reactions were carried out using a Thermo Sequenase fluorescent labelled primer cycle sequencing kit (Amersham International plc, England), and a Perkin Elmer model 310 DNA sequencer. The sequences obtained were compared with each other and sequences from the DNA data base. Identification of the clones was carried out based on sequence similarity and signature sequences for each genus.

1.2.8.Cloning of 16S rDNA of soil novel organisms

The sequenced positions of 16S rDNA were from 1112 to 1379 in the *Escherichia coli* numbering system. However, the sequenced nucleotides numbers, taken alone, were insufficient for phylogenetic analysis. Other primer sets were synthesized specifically to amplify the unusual 16S rDNA. The forward primers synthesized were AS11F (5'TTCGTCGACGGTTGATCC YGSCRGAGGC) which corresponds to positions (in *E.coli*) 11 to 23 of archaeal 16S rRNA; AS343F (5'TTCGTCGACTACGGG GCGCASCAGGC) which corresponds to positions 343 to 359; AS564F (5'AAC CGTCGACTGGGCCTAAAG-

CGYCCGTAGC) which corresponds to positions 564 to 584; and AS908F (5'AACCGTCGACAAAGGAATTGGCGGGGGAGCAC) which corresponds to positions 908 to 934. Reverse primers (complementary to 16S rRNA) were synthesized to be specific to the unusual 16S rDNA based on the determined partial 16S rDNA sequences. The reverse primers, which had sequence complementary to positions 1247 to 1229, were SC13 (5'CGGCGAATTCTCCACCATTGTTGCGCG) and SW42 (5'CGGCGAATTCCCTAT-CATTGTTGCGCG). These primers had Eco RI or Sal I restriction sites on the 5' end. PCR reaction and cloning were carried out as described above.

1.2.9.Phylogenetic analysis of soil novel clones

For phylogenetic analysis, sequences were initially aligned using the program Clustal W (version 1.7) [12], and manually realigned to remove some unalignable regions on domain level. Phylogenetic analyses were carried out by using nucleotides in the regions of which secondary structure was conserved among Eucarya, Bacteria and Archaea. The compared sequences were positions 632 to 820, 880 to 996, 1041 to 1132, and 1142 to 1229. The numbers of compared nucleotides were approximately 460. Maximum parsimony (MP) tree reconstruction was performed using PAUP (version 3.1.1) [13]. Applied ratio of transition and transversion was 2:1. A heuristic search was used with a random stepwise addition sequence of 100 replicates, tree-bisection-reconnection branch swapping, MULPARS option. A further analysis was run with 100 bootstrap replicates, each consisting of 10 random additional replicates. Maximum likelihood analysis (ML) was carried out using program package MOLPHY (version 2.3b3) [14]. Maximum likelihood distance matrix was calculated using NucML and initial neighbor joining tree was reconstructed by NJdist in the MOLPHY. Maximum likelihood tree was finally obtained using NucML with R (local rearrangement search) option based on the HKY model [15]. Local bootstrap probabilities (LBPs) was estimated by the resampling of estimated log-likelihood (RELL) method [16,17]. Neighbor joining (NJ) analysis was performed by using PHYLIP (version 3.572) [18]. DNADIST from this program package was used to create a distance matrix based on Kimura "2 parameter" model [19]. This distance matrix was used to construct NJ tree using the NJ algorithm from NEIGHBOR (PHYLIP 3.572) and bootstrap analyses utilized 1000 replicate data sets.

1.3.Results and Discussion for soil archaeal flora analysis

1.3.1 Methanogen-like clones isolated from paddy soils in Japan

Signature sequences specific to each genus were determined based on their alignment. The data for the genera *Methanococcoides*, *Methanocorpusculum*, *Methanohalophilus*, *Methanolobus*, *Methanopyrus*, *Methanosaeta*, *Methanothermus*, and *Methanospirillum* were limited (one sequence for each genus) which resulted in a reduction of the accuracy of identifications based on their signature sequences. Because of this, the identification of these species was also carried out by calculating sequence homologies between the clones and sequences from the DNA data base. A total of 100 clones were sequenced, many of which were shown to contain archaeal 16S rDNA. None of the clones contained 16S rDNA from eucaryotes or other bacteria. Among the archaeal clones, eleven did not appear to be methanogens because the sequence similarities between these clones and methanogen sequences were very low (lower than 84 %). Some of these clones were related to the

sequences of Crenarchaeota, which are known as extreme thermophiles or sulfur-dependent archaea [20]. Others were related to marine planktonic archaea [5]. Of the 100 clones, 89 were identified as methanogens. Of the methanogens, 54% (48 clones) had *Methanosarcina*-like sequences, 26% (23 clones) *Methanogenium*-like sequences, and 17% (15 clones) *Methanosaeta*-like sequences.

1.3.2.Soil novel clones

Three clones, SC13, SW42, and GA23, seemed to be phylogenetically very far from crenarchaeota and euryarchaeota. A representative species of crenarchaeota, *Sulfolobus solfataricus*, showed average sequence homology of 77.8% to euryarchaeotal species, and a representative species of euryarchaeota, *Methanobacterium thermoformicicum*, showed average sequence homology of 79.7% to crenarchaeotal species. The averages of sequence homology between the three clones and crenarchaeota were from 70.8 to 73.5%, and those between the three clones and euryarchaeota from 72.7 to 75.4%. Those values were smaller than the values between euryarchaeota and crenarchaeota. The primers used for amplification were designated for archaea, and no clones were identified as bacteria or eucarya. The three unusual clones also had archaeal signature sequences. Therefore, the three clones are presumed to belong to a very deep lineage of archaea.

Above determined sequences were too short to draw phylogenetic tree. Then, we tried to isolate longer clones. Primer sets of AS11F-SC13, AS11F-SW42, AS343F-SC13, and AS343F-SW42 did not amplify the unusual 16S rDNA. Primer sets of AS564F-SC13 and AS564F-SW42 amplified target DNAs from two soil samples collected at Yamaguchi and Kumagaya. However, 16S rDNA were amplified very clearly by primer sets of AS908F-SC13 and AS908-SW42 for all soil samples. Sequences of ten cloned DNAs were determined for futher analyses, and sequences of each clone were 82 to 98% similar to each other. The length of sequenced DNA ranged from 644 to 647 nucleotides. Each sequence formed secondary structure of 16S rRNA positions from 585 to 1228, and these sequences seemed not to be chimeras.

Phylogenetic analysis of the data sets by maximum parsimony, neighbor joining, and maximum likelihood all resulted in similar topologies. Finally we draw a tentatively conclusive phylogenetic tree (Figure 1). However, the topology of phylogenetic trees was not reliable when the bootstrap showed moderate to lower level. Deep branchings, such as those between bacterial phylums, usually show relatively low bootstrap, and the different branching topology was presented in various published papers, even though the same molecule was used for these analyses. We next tried to find out signature sequences to distinguish the novel soil clones from Archaea, referring to Woese's review [21]. The signature sequences are shown in Table 1. Marine planktonic clones were recognized by Fuhrman *et al.* [5]. Later, Mcinerney *et al.* [22] found clones belonging to the same lineage in an abyssal deposit feeder. Fuhrman *et al.* [5] reported that the planktonic clones belonged to the Crenarchaeotal lineage, but Mcinerney *et al.* [22] reported that the planktonic and abyssal clones formed a discrete phylogenetic cluster that was separated from Crenarchaeota and Euryarchaeota. Signature sequence analysis (Table 1) revealed that the signature nucleotides of planktonic and abyssal clones and "Korarchaeota" were identical to those of Archaea. In contrast, the novel soil clones showed signature sequences that appeared to be different from those of Archaea (including planktonic and abyssal clones and "Korarchaeota"), Bacteria, and Eucarya. The differences that distinguished the novel soil

clones from Archaea, Bacteria, and Eucarya were found in positions 688, 693, 699, 946, 1050, 1114, 1186, 1208, and 1235. Overall signature sequences seemed to show closer relationships to Archaea and Eucarya than to Bacteria. Signature sequences that showed close relationships to Archaea and Eucarya were positions 716, 912, 931, 952, 966, 1086, 1109, and 1110. The average sequence homology of novel soil clones to Crenarchaeota and Euryarchaeota was 78.0%, a value much lower than the average value (83.7%) between Crenarchaeota and Euryarchaeota.

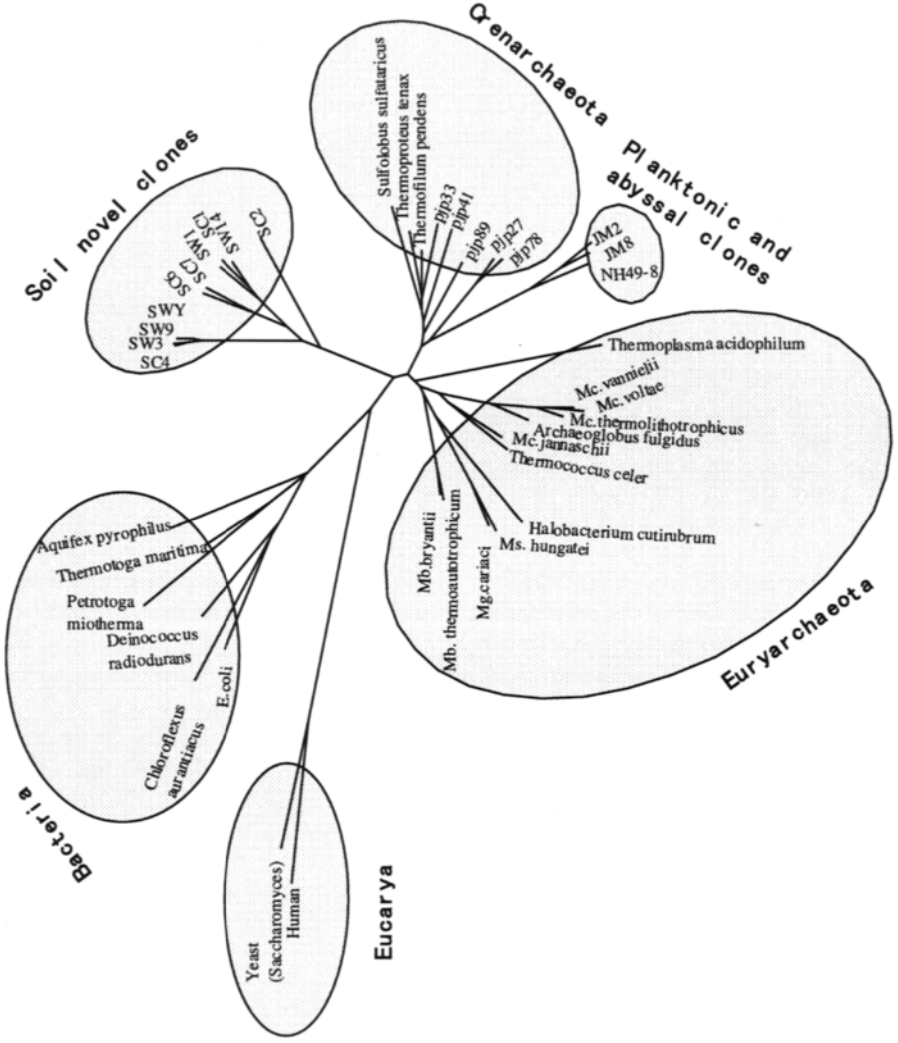

Figure 1. Phylogenetic tree of life including soil novel clones.

Table 1

16S rRNA sequence signature distinguishing Bacteria, Eucarya, Archaea, planktonic and abyssal clones, and soil novel clones.

Position	Novel Soil	Archea*	Eucarya	Bacteria
688	C	G	G	G
693	A	R	G	mostly G
699	G	Y	C	C
716	C	C	Y	A
756	G	G	mostly A	mostly C
912	U	mostly U	U	mostly C
931	G	G	mostly G	C
946	G	A	A	A
952	C	C	C	U
962	Y	G	U	mostly C
966	U	U	U	G
973	R	C	G	mostly G
975	mostly A	G	G	mostly A
1050	A	G	G	G
1086	C	mostly C	C	mostly U
1087	C	C	mostly U	mostly G
1109	A	A	A	C
1110	G	G	G	A
1114	U	C	Y	C
1186	A	G	mostly A	G
1208	mostly U	C	mostly C	C
1235	A	U	U	U

*includings planktonic abyssal clones and "Korarchaeota"

Following the proposal of Archaebacteria as a category, most biologists and microbiologists havecome to accept a life systematics with three major lineages. Subsequently, discussions on the early evolution of life focused on the possible origins of eukaryotes. Although the small subunit ribosomal RNA could not show the close relationship between Archaea and Eucarya, the close relationship of the two domains was gradually realized from various evidences, such as similarity in second largest subunit of RNA polymerases [23], transcriptional machinery [24], elongation factor Tu and G [25], a U3-like RNA in rRNA processing [26], and presence of histone-like protein [27]. The composite phylogenetic tree of primordially duplicated proteins conclusively demonstrated the close relationship [23,28]. Accordingly, the three domain system, which describes close relationships between Archaea and Eucarya, was proposed [20,29], and this system has been widely accepted. However, a conflicting system was proposed by Lake and his colleagues [30,31] before the proposal of three domain system. The Lake's category of eocyte was established based on the morphology of ribosome [31] and was very often denied [32]. Lake and colleagues [30,31] insisted that the eocyte, which is equal to the crenarchaeota, would be ancestral lineage of eukaryotes. The category of eocyte still gains

its standpoint because the peptide sequences of several proteins place the eocyte in the ancestral position of the Eucarya [23,25,33]. If the novel soil clones are placed in an intermediate position between Archaea and Eucarya, the organisms from which the clones derived will be vital to any discussion of the origin of eukaryotes.

1.3.3.Image of soil novel organisms

It is very difficult to imagine the new lineage of life. However, we can draw its faint image from the G+C content of 16S rDNA, and from the data on natural distribution. The Crenarchaeota showed higher G+C contents than the other groups. High G+C content of the Crenarchaeota probably related to its thermophilic characteristic. The Euryarchaeota is comprized mostly of mesophilic species, and its members showed lower G+C contents than Crenarchaeota. Judging from the G+C contents of novel soil clones, the novel organisms would be supposed mesophilic. In this paper and in a previous paper [34], we reported that the ratio of novel soil clones in Japanese paddy soil to total methanogen clones was 3.4%. The cell numbers of methanogens per gram of wet soil had been reported in the range of 10^5 to 10^6 [35]. Therefore, supposing that the clone analysis was proportionate to the cell number of novel soil organisms and methanogens, one gram of soil contains at least 10^3 cells of the novel soil organisms. In order to examine distribution of the novel organisms in the environments, we designed an oligonucleotide primer 970F (5'AATYYAA-CTCAACGCRGAG) having a sequence that is identical to positions 959 to 976 of 16S rRNA of the novel organisms. The presence of the novel soil organisms was tested with a primer set of 970F and SC13 for paddy soils, upland soils, and forest soils collected in Japan. The result showed that the novel organisms were detected in all of paddy soils, but in none of upland and forest soils, suggesting that the novel soil organisms are anaerobic.

In the present study we discovered a novel phylogenetic cluster of life in the soil DNAs. This phylogenetic cluster showed a unique signature sequence in 16S rDNA. The cluster was also diverse phylogenetically. We have not yet recognized the organisms from which the novel clones derived but existence of soil novel microorganisms were detected in paddy by *in situ* hybridization. Detected cell was cocci and many cells were not detected because of impurities of soil. Soil novel organisms specific probes were not bound to Archaea. Only Archaea specific probe in mixed primers was bound to pure cultured test strains. Although detected cells were very few, however, the organisms are presumed to inhabit paddy soils ubiquitously, and average frequency of approximately 10^3 to 10^5 cells per g wet soil, and this would indicate an important role in the soil anaerobic ecosystem. If these organisms are cultured, they will one day provide useful information of not only on microbial ecology, but also on the early evolution of the Eucarya.

2.GROUP TARGETED CLONE ANALYSIS OF ENHANCED BIOLOGICAL PHOSPHATE REMOVAL SLUDGE

2.1.Background about enhanced biological phosphate removal processes

Alternating anaerobic-aerobic treatment on the activated sludge has been known to remove phosphorous from wastewater, which is called enhanced biological phosphate removal processes (EBPR). Many studies have been carried out to reveal the key microorganisms which play an important role in the EBPR [36-40]. Studies with isolation techniques

suggested that the members of *Acinetobacter* spp. were the dominant microorganisms in the EBPR [41,42]. However, there were critical discrepancies to link the EBPR and the members of *Acinetobacter* species. The most critical is that the carbon and phosphorus transformation patterns of *Acinetobacter* species are not consistent with those of the EBPR sludge [43,44].

The cultivation on the agar media is highly selective and never reveals real microbial structure. Then, in recent years, culture-independent techniques such as *in situ* hybridization [36,45-47], clone analysis [48,49], quinone profile analysis [40,46], and denaturing gradient gel electrophoresis analysis (DGGE) or thermal gradient gel electrophoresis analysis (TGGE) [50] have been applied to the EBPR microbial community. These culture-independent studies showed that members of *Acinetobacter* species never play an important role in the EBPR processes. Recent studies strongly suggested that the Gram positive with high G+C bacteria and alpha proteobacteria are performing phosphate accumulation [46,47]. In this study we carried out clone analyses targeted to Gram-positive high GC bacteria and alpha proteobacteria, and try to get some information about bacterial community of EBPR sludge.

2.2.Material and methods for group targeted clone analysis of enhanced biological phosphate removal sludge

2.2.1.DNA preparation from the EBPR sludge

The sample was obtained from a laboratory scale continuous anaerobic-aerobic activated sludge process. The reactor was fed with synthetic wastewater containing acetate, propionate, peptone, and yeast extract as the main carbon sources. The anaerobic retention time and the aerobic retention times were 1.9 hour and 4.6 hour respectively, and the sludge retention time was 10 days. Return sludge ratio was 100%. Mixed liquor suspended solid was around 4000 mg/l, and the phosphorus content was around 4 to 5 percent of the mixed liquor suspended solid. DNA was extracted from 100 mg of wet EBPR sludge by the method described by Zhu *et al.* [11]. The crude DNA was purified with QIAGEN-tip 20 (QIAGEN Inc. Germany).

2.2.2.Primers and PCR condition

Primers for Gram positive high GC group were Hgc-1, 5'TTCGGGTTGTAAACCTCTTTC3', which corresponds to positions 420 to 440, and Hgc-2, 5'CCCCRATCCG-AACTGAGACCG3', which was complementary to positions 1292 to 1312. Primers for alpha proteobacteria were ALF1, 5'TTGATCCTGGCTCAGARC3', which corresponds to positions 13 to 30, and ALF2, CGAATTTCACCTCTACACTC3', which was complementary to positions 700 to 719. PCR condition was same as that described in 1.2.6.

2.2.3.Group targeted clone analysis of EBPR sludge

Amplified 16S rDNA was directly cloned into T vector pt-Adv (Clontech). For Gram positive high G+C group approximately 100 colonies and for alpha proteobacteria approximately 50 colonies were picked up and sequenced. Sequencing reactions were carried out by the methods described in 1.2.7.

2.2.4.Phylogenetic analysis

For phylogenetic analysis, sequences were initially aligned using the program Clustal W (version 1.7) [12], and manually realigned to remove some unalignable regions. Neighbor joining (NJ) analysis was performed for Gram positive and alpha proteobacterial clones by using PHYLIP (version 3.572) [18]. DNADIST from this program package was used to create a distance matrix based on Kimura "2 parameter" model [19]. This distance matrix was used to construct NJ tree using the NJ algorithm from NEIGHBOR (PHYLIP 3.572).

2.3.Results and discussion for group targeted clone analysis of enhanced biological phosphate removal sludge

Phylogenetic trees were drawn in Figure 2 (Gram positive bacteria with high G+C contents) and Figure 3 (alpha proteobacteria). All of sequences retrieved from PCR products amplified with Gram-positive high GC primers and alpha proteobacteria primers were included in respective expected clusters in a phylogenetic tree. Of 40 Gram-positive high GC clones included in Figure 2, 35 clones were closely related to *Dermatophilus congolensis*, two clones to *Terrabacter* species, two to *Archanobacterium*, and one to *Mycobacterium-Nocardia* cluster. *Terrabacter* species and *Dermatophilus congolensis* were on very near clusters. Therefore, 37 of total 40 clones were distributed in a very close cluster. Our result for Gram-positive high GC bacteria well coincided with those reported by Christensson *et al.* [49]. Clones of *Microlunatus phosphovorus*, which was suspected as polyphosphate accumulating organisms (PAOs) [49,51,52], were not obtained in this study. Kawaharasaki *et al.* reported that the cell numbers of *Microlunatus* in the EBPR sludge detected by FISH method were rather small [47]. Therefore, our result of clone analysis is consistent with the observation by Kawaharasai *et al.* The sequences of nineteen clones of alpha proteobacteria were included in Figure 3. These clones distributed widely in alpha proteobacterial clusters. Seven clones were closely related to the spiral bacteria, *Azospirillum brasilense*, *Rhodocista centenaria*, and *Rhodospirillum rubrum*, and five to the members of genera *Paracoccus*, *Roseobacter*, *Amaricoccus*, *Tetracoccus* and *Silicibacter*, four to *Rhizobium* species, and one to *Caulobacter* species. Clone 2 and 11 formed a cluster separated from the other major clusters of alpha proteobacteria.

Morphological characteristics of PAOs and Glycogen accumulating bacteria (G bacteria) has been studied by selective staining methods. It was revealed that very short and mostly irregular rods stained Gram positive or negative are accumulating polyphosphate in the cells [46,47]. Tetrad-arranged cocci do not accumulate polyphosphate and are suspected to be G bacteria [46]. One of the tetra coccus was isolated and classified as *Tetracoccus cechii* [53]. As mentioned above 35 of total 40 Gram positive high GC clones were closely related to *Terrabacter* species and *Dermatophilus congolensis*. Cells of *Terrabacter* are very short irregular rods which seems to be cocci. Cells of *Dermatophilus congolensis* are also very short rods forming chains. The dominance of these clones in the Gram-positive high GC clone library and morphological characteristics of cultivated close relatives leads to the idea that the organisms would be PAOs. The close relatives of tetrad-arranged cocci, *Tetracoccus cechii*, belonged to one of the dominant groups in the alpha proteobacterial clone library, and they are suspected to be G bacteria. The most dominant clones in the alpha proteobacterial clone library are close relatives of spiral bacteria. Spiral bacteria are not dominated in the EBPR sludge. However, *Rhodocista centenaria* and most of the members

of the genus *Azospirillum* are very short spirals which sometime seems to be irregular rods. Therefore, the close relatives of spiral alpha proteobacteria are also suspected to be PAOs.

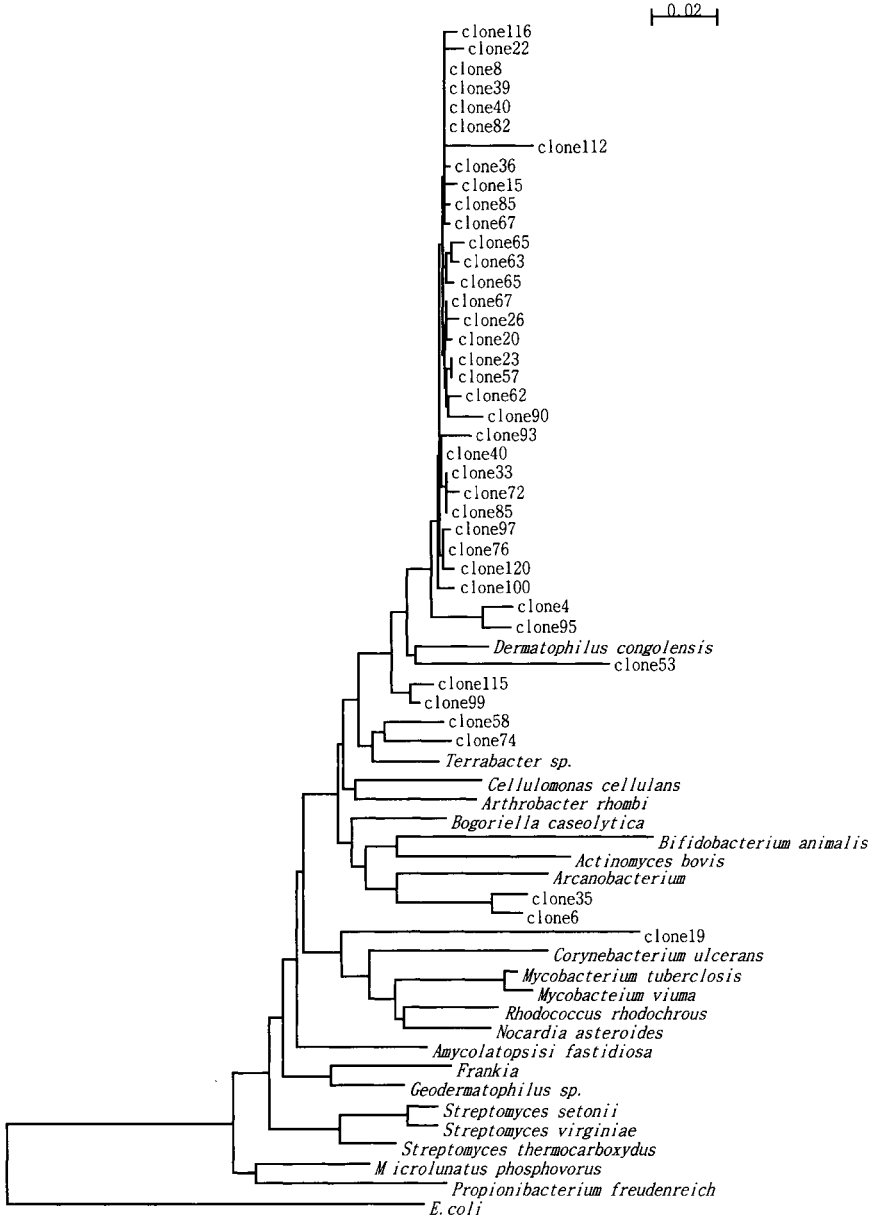

Figure 2.Phylogenetic tree for Gram positive high GC clones obtained from EBPR sludge

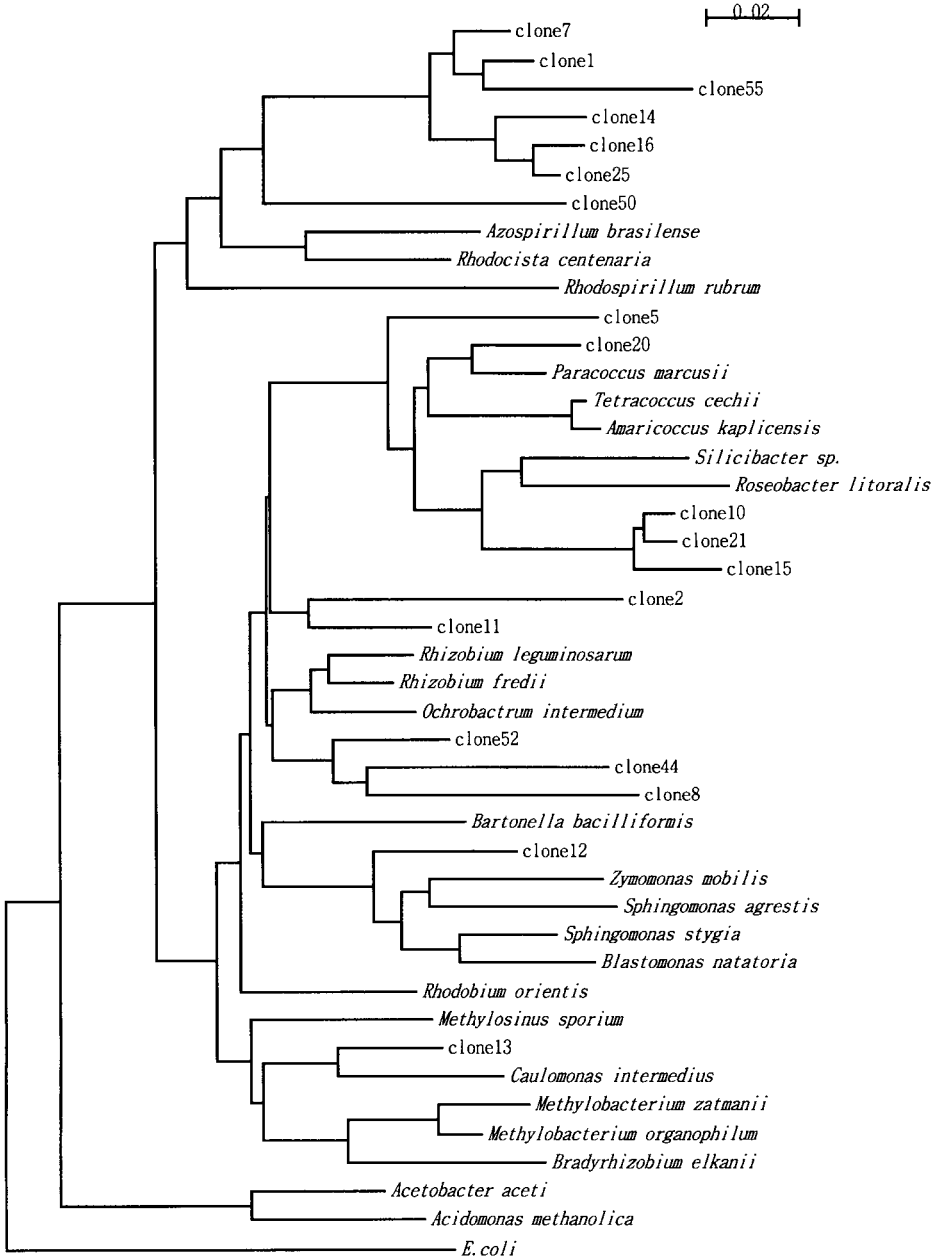

Figure 3.Phylogenetic tree for alpha proteobacterial clones obtained from EBPR sludge

REFERENCES

1. H. Oyaizu, In "Biseibutunoseitai 18," ed. by Gakkaishuppan center Co., Tokyo (1992) 51.
2. D. M. Ward, R. Weller, J. Shiea, R. W. Castenholz, and Y. Cohen, Nature, 345 (1990) 63.
3. D. M. Ward, M. M. Bateson, R. Weller, and A. L. Ruff-Roberts, In "Advances in Microbial Ecology vol. 12," ed. by K.C. Marshall, Plenum Press, New York (1992) 219.
4. R. Weller and D. M. Ward, Appl. Environ. Microbiol., 57 (1991) 1146.
5. J. A. Fuhrman, K. McCallum, and A. A. Davis, Nature, 356 (1992) 148.
6. E. F. Delong, Proc. Natl. Acad. Sci. USA, 89 (1992) 5685.
7. S. M. Barns, C. F. Delwiche, J. D. Palmer, and N. R. Pace, Proc. Natl. Acad. Sci. USA, 93 (1996) 9188.
8. S. M. Barns, R. E. Fundyga, M. W. Jeffries, and N. R. Pace, Proc. Natl. Acad. Sci. USA, 91 (1994) 1609.
9. S. Asakawa, H. Morii, M. Akagawa-Matsushita, Y. Koga, and K. Hayano, Int. J. Syst. Bacteriol., 43 (1993) 683.
10. S. Asakawa, M. Akagawa-Matsushita, H. Morii, Y. Koga, and K. Hayano, Curr. Microbiol., 31 (1995) 34.
11. H. Zhu, F. Qu, and L.-H. Zhu, Nucleic Acid Research, 21 (1993) 5279.
12. J. D. Thompson, D. G. Higgins, and T. J. Gibson, Nucleic Acids Res., 22 (1994) 4673.
13. D. L. Swofford, PAUP:Phylogenetic analysis using parsimony. Version 3.1, Illinois National History Survey, Champaign, Illinois (1991).
14. J. Adachi and M. Hasegawa, Computer Science Monographs, No 28, MOLPHY: Version 2.3. Programs for molecular phylogenetics based on maximum likelihood. Institute of Statistical Mathematic, Tokyo, 1996.
15. M. Hasegawa, H. Kishino, and T. Yano, J. Mol. Evol., 22 (1985) 160.
16. H. Kishino, T. Miyata, and M. Hasegawa, J. Mol. Evol., 31 (1990) 151.
17. M. Hasegawa and H. Kishino, Mol. Biol. Evol., 11 (1994) 142.
18. J. Felsenstein, PHYLIP Version 3.57c. University of Washington, Seattle, 1995.
19. M. Kimura, J. Mol. Evol., 16 (1980) 111.
20. C. R. Woese, O. Kandler, and M. Wheelis, Proc. Natl. Acad. Sci. USA, 87 (1990) 4576.
21. C. R. Woese, Microbiol. Rev., 51 (1987) 221.
22. J. O. Mcinerney, M. Wilkinson, J. W. Patching, T. M. Embley, and R. Powell, Appl. Environ. Microbiol., 61 (1995) 1646.
23. N. Iwabe, K. Kuma, H. Kishino, and M. Hasegawa, J. Mol. Evol., 32 (1991) 70.
24. P. Baumann, S. A. Qureshi, and S. P. Jackson, Trends Genet., 11 (1995) 279.
25. S. L. Baldauf, J. D. Palmer, and W. F. Doolittle, Proc. Natl. Acad. Sci. USA, 93 (1996) 7749.
26. S. Potter, P. Durovic, and P. P. Dennis, Science, 268 (1995) 1056.
27. M. R. Starich, K. Sandman, J. N. Reeve, and M. F. Mummers, J. Mol. Biol., 255 (1996) 187.
28. N. Iwabe, K.-I. Kuma, M. Hasegawa, S. Osawa, and T. Miyata, Proc. Natl. Acad.Sci. USA, 86 (1989) 9355.
29. M. L. Wheelis, O. Kandler, and C. R. Woese, Proc. Natl. Acad. Sci. USA, 89 (1992) 2930.
30. J. A. Lake, Trends Biochem. Sci., 16 (1991) 46.
31. J. A. Lake, E. Henderson, M. Oakes, and M. W. Clark, Proc. Nat. Acad. Sci. USA, 81 (1984) 3786.
32. M. Gouy and W.-H. Li, Nature, 339 (1989) 145.
33. M. C. Rivera and J. A. Lake, Science, 257 (1992) 74.
34. Y. Kudo, T. Nakajima, T. Miyaki, and H. Oyaizu, FEMS Microbiol. Ecol., 22 (1996) 39.
35. S. Asakawa and K. Hayano, Biol. Fertil. Soils, 20 (1995) 113.
36. M. Wagner, R. Erhart, W. Manz, R. Amann, H. Lemmer, D. Weedi, and K. H. Schleifer, Appl. Environ. Microbiol., 59 (1993) 1520.

37. M. C. Wentzel, L. H. Lotter, R. E. Loewenthal, and G. v. R. Marais, Water SA, 12 (1986) 209.
38. M. Streichan and J. R. Golecki, FEMS Microbiol. Ecol., 73 (1990) 113.
39. K. Nakamura and M. Dazai, J. Ferment. Technol., 64 (1986) 433.
40. A. Hiraishi, K. Masamune, and H. Kitamura, Appl. Environ. Microbiol., 55 (1989) 897.
41. G. W. Fuhs and M. Chen, Microbiol. Ecol., 2 (1975) 119.
42. L. H. Lotter and M. Murphy, Water SA, 11 (1985) 179.
43. H. Ohtake, K. Takahashi, Y. Tsuzuki, and K. Toda, Wat. Res., 19 (1985) 1587.
44. J. W. Groenestijn, M. Zuidema, J. J. M. vandeWorp, M. H. Deinema, and A. J. B. Zehnder, Antonie Van Leeuwenhoek, 55 (1989) 67.
45. M. Wagner, R. Erhart, W. Manz, R. Amann, H. Lemmer, D. Wedi, and K.-H. Schleifer, Appl. Environ. Microbiol., 60 (1994) 792.
46. I. M. Sudiana, T. Mino, H. Satoh, and T. Matsuo, Wat. Sci. Tech., 38 (1998) 69.
47. M. Kawaharasaki, H. Tanaka, T. Kanagawa, and K. Nakamura, Wat. Res., 33 (1999) 257.
48. P. L. Bond, P. Hugenholtz, J. Keller, and L. L. Blackall, Appl. Environ. Microbiol., 61 (1995) 1910.
49. M. Christensson, L. L. Blackall, and T. Welander, Appl. Microbiol. Biotechnol., 49 (1998) 226.
50. A. T. Nielsen, W.-T. Liu, C. Filipe, J. L. Grady, S. Molin, and D. A. Stahl, Appl. Environ. Microbiol., 65 (1999) 1251.
51. K. Nakamura, K. Matsuda, and E. Mikami, J. Ferment. Bioeng., 71 (1991) 258.
52. K. Nakamura, A. Hiraishi, Y. Yoshimi, M. Kawaharasaki, K. Matsuda, and Y. Kamagata, Int. J. Syst. Bacteriol., 45 (1995) 17.
53. L. L. Blackall, S. Rossetti, C. Christensson, M. Cunningham, P. Hartman, P. Hugenholtz, and V. Tandoi, Lett. Appl. Microbiol., 25 (1997) 63.

Microbial community analysis of thermophilic contact oxidation process by using PCR-DGGE method

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A lab scale thermophilic aerobic wastewater treatment reactor was operated using peptone and starch as primary carbon sources, and the microbial community was analyzed mainly by denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rDNA partial sequences (PCR-DGGE). The temperature began to rise on day 2 and varied cyclically between 30-65 °C after each batch feeding from day 10. PCR-DGGE indicated changes in the community profile, which was almost unchanged after day 23. DGGE bands were further excised and sequenced to identify the species of the DGGE bands. According to the quantification of DGGE bands intensity, population dynamics of those species are discussed. Gram positive bacteria with low G+C content predominated since day 2 and among that, *Bacillaceae* was probably dominant. Relatives of *B.licheniformis, B.pumilis* and *B.thermocloacae* were abundant during the stable state. Interestingly, not only facultative thermophilic *Bacillus* but also obligate thermophilic and mesophilic *Bacillus* seemed to appear in the reactor during the stable state, in spite of the temperature variation.

1. INTRODUCTION

Thermophilic contact oxidation process (TCOP) is a novel and unique process to treat high strength organic wastewater. TCOP can achieve oxygen supply for aerobic degradation, which is difficult for highly concentrated wastewater, by absorbing wastewater to water absorbent media, such as wood chips. Organic compounds of the wastewater are aerobically degraded on or in the wood chips. Another characteristics of TCOP is extremely high ratio of degradation. More than 90% of organic matter can completely be degraded into carbon dioxide [1]. Moreover, water is evaporated due to the aeration under high temperature because of the degradation heat.

Microbiology in this process has not been clarified yet. Thus, it is not known yet how and why such high degradation ratio which is unusual in the normal aerobic degradation can be

achieved. In addition, the reason why operation of the process sometimes fails due to no temperature rise must be elucidated for further application.

Molecular biological techniques are suitable to analyze whole microbial communities. Among them, denaturing gradient gel electrophoresis (DGGE) of PCR-amplified gene fragments coding for 16S rRNA (PCR-DGGE method) has recently been utilized to determine the genetic diversity of natural microbial communities [2,3]. By using DGGE, DNA fragments of the same length but with different base pair sequences, such as PCR fragments obtained from a mixed culture, can be separated. Similarity of microbial communities can be analyzed from electrophoresis patterns [2] and bands can further be excised from gels and sequenced to identify the phylogenetic affiliation of the community members [4].

In the present study, A bench scale TCOP reactor was operated using peptone and starch as primary carbon sources and microbial community was analyzed mainly by PCR-DGGE method. It was utilized to determine population dynamics at species/strain level. DGGE bands were excised and sequenced to elucidate species that constituted the community. According to the results of community analysis, community structure and its variation were assessed in relation to process performance.

2. MATERIALS AND METHODS

2.1. Operation and monitoring of TCOP reactor

The experimental set-up is shown in Figure 1. The working volume of the reactor was 10 liters, and 6 liters of wood chips sized 2-4 mm were installed as media. To maintain a constant rate of heat loss, the reactor was placed in a styrofoam box and the temperature outside the reactor was controlled at 4 ^{o}C lower than inside it. The media were mixed slowly and continuously by the mixing blades in the reactor.

The reactor was fed with 300 ml of the synthetic wastewater every other day. The synthetic wastewater contained 82 g/l soluble starch, 82 g/l peptone, 41 g/l sucrose and 41 g/l

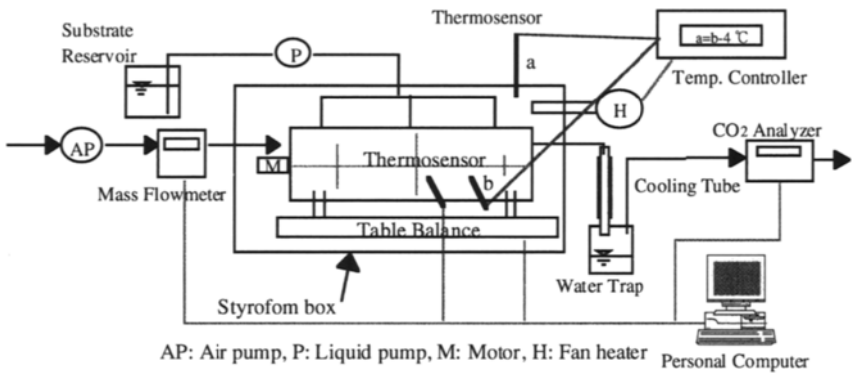

Figure 1. The experimental set up of the reactor.

yeast extract, and its carbon and nitrogen content was 11 gC/l and 1.6 gN/l, respectively. At the beginning of the operation, 600 ml of the synthetic wastewater was added to the reactor and mixed with the media. Then, the reactor was inoculated with 60 g (dry weight) of semi-aerobic compost. The reactor was aerated at the rate of 1.8-2.0 l/min (300-370 l/[m^3 chip]/min). The aeration rate was measured precisely by a mass flowmeter. Carbon dioxide concentration in emission gas was monitored by CO_2 meter (LX-710, Iijima electronics, Aichi, Japan).

2.2. DNA extraction and PCR amplification

Samples for community analysis were taken from the reactor at the times shown in Figure 2. Half gram of the samples were immersed in 5 ml sterilized phosphate-buffered saline (1xPBS; pH7.2) and the immersion was sonicated for 5 min at 15 W (Branson Sonifier 450, Krautkramer-Branson, Penn., USA). The suspension was filtrated with a coarse filter paper (No.1, Advantec-Toyo, Tokyo, Japan) to remove large particles and stored in liquid nitrogen until use.

One milliliter of suspended samples were washed with TE buffer and genomic DNA was extracted with benzyl chloride followed by ethanol precipitation as described by Zhu *et al.*[5]. Primers complementary to conserved regions were used to amplify a 194-bp fragment of the 16S rDNA corresponding to nucleotides 341 to 534 in the *E.coli* sequence [3]. The nucleotide sequence of the forward primer, which is specific to eubacteria (357f; 5'-CCTACGGGAGGCAGCAG-3'), contains at its 5' end a 40-base GC clamp (357fGC; 5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCAG-3') [3]. The universal consensus sequence was used as a reverse primer (518r; 5'-ATTACCGCGGCTGCTGG-3'). PCRs were performed by using AmpliTaq Gold DNA polymerase (Applied Biosystems, Perkin Elmer Japan, Tokyo, Japan) according to the instructions provided by the manufacturer. Thermalcycler we used was TaKaRa Thermalcycler (Takara Biotechnology, Tokyo, Japan). PCR cycling parameters are as follows. PCR mixtures were preincubated at 94 °C for 9 min to activate DNA polymerase. Denaturing was carried out at 94 °C for 1 min, annealing was performed at 53 °C for 1 min, and extension was performed at 72 °C for 2 min. This cycle was repeated for 35 times, and then incubated at 72 °C for 10 min for the final elongation. All PCR products were analyzed by electrophoresis with 4% (w/v) agarose gels before DGGE analysis was performed.

2.3. Analysis of PCR products by DGGE

DGGE was performed according to Muyzer *et al.* [3] with small modifications. Before loading to the DGGE gel, PCR products were incubated at 95 °C for 5 min, at 65 °C for 1 h and gradually cooled to room temperature to avoid non-complementary annealing of DNA. Gels for DGGE were 8% polyacrylamide gel (8% acrylamide and N,N-methylene-bisacrylamide solution [37.5:1 {v/v}], x% [v/v] formamide, y M urea and 1x TAE) containing a linear gradient of the denaturant concentration ranging from 35% to 50%. Denaturant concentration of 100% corresponds to x=40 and y=7. Denaturing gradient gels were prepared by using Model 475 gradient delivery system (Bio-Rad Laboratories, Calif., USA). The gels were run by D Code system (Bio-Rad) for 300 min at 60 °C and 130V.

After completion of electrophoresis, the gels were stained with Vistra Green (Amersham Pharmacia Biotech, Tokyo, Japan) by spreading the staining solution on the gel for 15 minutes, and documented by fluorescent image scanner (Fluorimager 595, Molecular Dynamics, Calif., USA). Intensely stained bands were excised from the gels and soaked into 50μl of sterilized water. DNA was recovered from the gels by freeze-thawing more than 3 times.

2.4. Similarity analysis of DGGE banding patterns

DGGE banding patterns were compared to each other by using the dissimilarity index, which was calculated as follows. Fluorescent intensity of the bands was quantified by using the ImagequaNT tool provided with Fluorimager 595. The ratio of the intensity of a band to the sum of the intensity of all the bands in a sample was defined as 'relative intensity of the band'. Bands that had the same location among different samples were assumed to have the same sequence and named with the same band numbers. The dissimilarity index [6] was calculated for every two samples as follows:

$$D(i,j)=1/2\Sigma \mid xik - xjk \mid , \quad (1)$$

where

$$\Sigma xik = \Sigma xjk = 1 \quad (2)$$

and xik and xjk are the relative intensity of the band *k* in the sample *i* and *j*, respectively. If a band appeared only in one sample, the relative intensity of the other band was defined as zero. Clustering of each sample by the group average linkage method was performed by NEIGHBOR tools in the software package PHYLIP 3.5 based on the dissimilarity index.

2.5. Sequencing

DNA fragments recovered from the DGGE bands were reamplified with the forward primer 357f including an additional sequence extension (T3; 5'-AAAATTAACCCTCACTAAAG-3') at its 5' end and the reverse primer 518r with an extension (M13r; 5'-AAATTCACACAGGAAACAG-3') at its 5' end to facilitate DNA sequencing [4]. PCR conditions were all the same as the first amplification except the cycle number. The cycle number was reduced to 25 times to minimize the amplification of contaminants. The newly obtained PCR products were directly sequenced with SQ-5500 automatic sequencing system (Hitachi, Tokyo, Japan). Sequencing reactions were done by using the Vistra Sequencing Kit (Amersham) with T3 and M13r primers labeled with Texas Red according to the instruction provided by Amersham and Hitachi.

3. RESULTS

3.1. Performance of the reactor

Temperature in the reactor throughout the operation is shown in Figure 2. The temperature reflects bacterial activity very well because it rises due to the heat by the decomposition of organic materials. The temperature started rising after two days, and on the day 4, it reached 65 °C. As the mixing motor stopped from day 4 to 6 due to an accident, the temperature did not rise during this time because of the lack of oxygen supply. The temperature started rising again after the recovery of the mixing, and it changed periodically from day 30 to day 70. After day 70, temperature rise deteriorated gradually and from day 80, no temperature rise occurred.

A carbon balance was performed on the system using the degradation ratio, which was defined as the ratio of CO_2-carbon discharged in two days divided by organic carbon fed in a batch. It was 40 to 100% from day 20 to day 58 (Figure 3). After day 58, no data was available because the CO_2 meter was broken. Degradation of organic matter accumulated in the reactor may result in the values over 100%. About 80% of total organic carbon input was converted to CO_2 and discharged from the reactor by day 58.

3.2. PCR-DGGE analysis

Figure 4 shows the result of the PCR-DGGE analysis. The banding pattern of day 0, i.e. inoculum, was completely different from that of day 1. The patterns were quite simple until day 8, but became diverse after day 11. From day 23, they seemed to become stable until the end of the operation. Similarity analysis of the banding pattern was conducted and the result is shown as cluster analysis (Figure 5). They could be classified into 3 clusters: 1) day 1 to 8, 2) day 11 and 15, and 3) day 23 to 87. The band pattern of day 0 (inoculum) was excluded from similarity analysis because it was completely different from that of day 1 and no band was at the same location.

3.3. Sequencing of DGGE bands

Prominent DGGE bands from the samples on day 4 and day 31 and some other distinctive bands were sequenced to elucidate the affiliation of constituents of the communities. Table 1 shows the results of homology search for the sequences of the DGGE bands. Three bands, SP31-2, SP4-3 and SP4-8, have completely identical sequences (100% homology) with the described species, minimum homology was 92% and average was 96.3%. By judging from the location of the bands, bands at the same location of those sequenced were assumed to have the same sequences and summarized in the table. Actually, several sets of bands with the same location were sequenced and all of them had the identical sequences. Moreover, in order to facilitate the analysis, we classified the bands into 8 groups in accordance with their similarity (Table 1).

3.4. Population dynamics in DGGE banding patterns

Intensity of the bands were measured, summed up in each bacterial group 1 to 8 in Table 1. The percentage of the groups to the sum of all visible bands were calculated. Figure 6

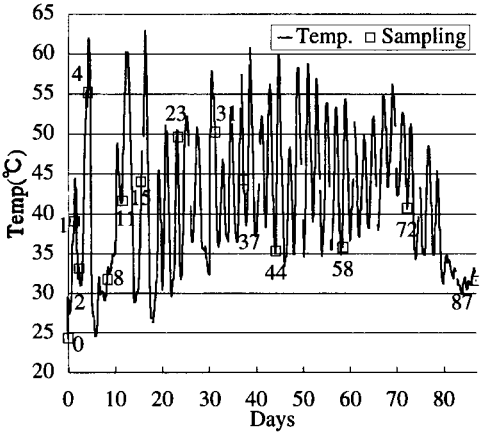

Figure 2. Temperature inside the reactor.The day samples were taken is indicated inthe figure.

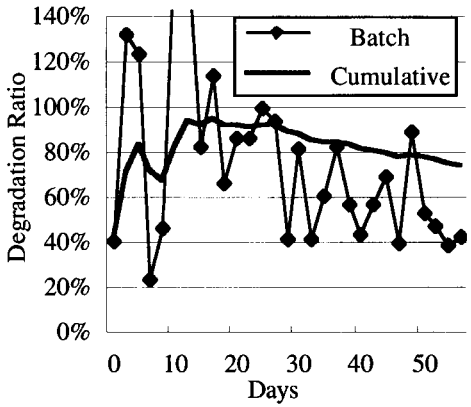

Figure 3. Degradation ratio in a batch and cumulative ratio of discharge and feeding. The ratio is based on carbon balance.

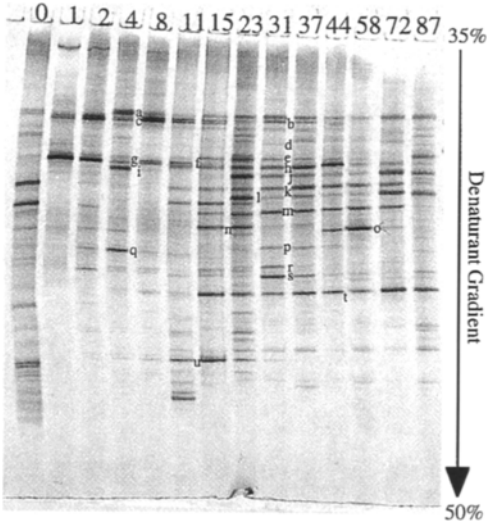

Figure 4. A gel image of PCR-DGGE analysis. Bands sequenced in this study are indicated with the No. 'a' to 'u' at the right side of the bands.

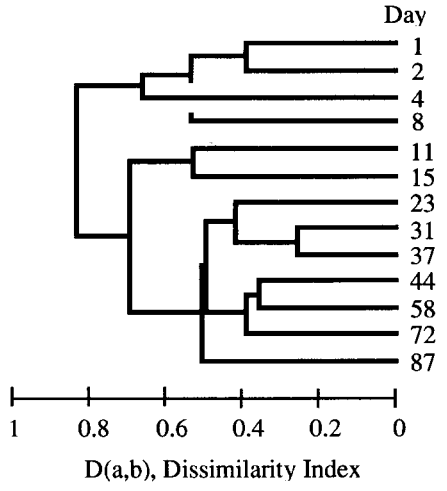

Figure 5. Similarity analysis of DGGE band pattern shown in Figure 6. Clusters are calculated by UPGMA method.

Table 1
Sequencing and identification of bands and their fate. The DGGE lanes, i.e.sampling date, which have a band at the same location as the sequenced bands, are marked'+'. The bands are classified into 8 groups according to their similarities.

Bands		Day														
No.	Name	1	2	4	8	11	15	23	31	37	44	58	72	87	Highest similarity[a]	Group
a	SP4-1			+	+	+	+	+	+	+	+	+	+	+	*B.oleronius*	1
b	SP31-2	+	+	+	+	+	+	+	+	+	+				*Staphyl.sciuri*	7
c	SP4-3	+	+	+	+	+	+				+				*Eco.saccharolyticus*	8
d	SP31-4			+					+					+	*B.licheniformis*	1
e	SP31-5			+		+	+	+	+	+	+	+			*B.firmus*	-
f	SP11-5				+	+									*Mcc.carouselicus*	-
g	SP4-8	+	+	+	+						+	+	+	+	*Lcc.lactis*	6
h	SP31-6					+	+	+	+	+					*B.licheniformis*	1
i	SP4-9	+	+	+	+										*Scc.thermophilus*	1
j	SP31-7							+	+	+	+	+	+	+	*B.thermocloacae*	2
k	SP31-8			+		+	+	+	+	+	+	+	+	+	*B.licheniformis*	1
l	SP23-11							+	+	+	+	+	+	+	*B.licheniformis*	1
m	SP31-10						+	+	+	+	+	+	+	+	*B.licheniformis*	1
n	SP15-10						+	+	+	+				+	*B.lentus*	4
o	SP58-10	+	+					+			+	+	+		*B.benzoevorans*	3
p	SP31-12							+	+	+	+	+			*B.thermocloacae*	2
q	SP4-13	+	+	+	+										*B.badius*	3
r	SP31-13							+	+	+					*B.thermocloacae*	2
s	SP31-14	+	+			+	+	+	+	+	+	+	+	+	*B.cohnii*	3
t	SP44-15				+	+	+	+	+	+	+	+	+	+	*B.pumilus*	3
u	SP11-17			+	+	+	+	+	+						*B.fastidiosus*	5

[a] *B.* : *Bacillus, Staphyl.* : *Staphylococcus, Eco.* : *Enterococcus, Mcc.* : *Macrococcus, Lcc.* : *Lactococcus, Scc.* : *Sacharococcus.*

shows the fate of each group by the band intensity. Group 1, *B. licheniformis* and its relatives, predominated after day 8. The profiles of the groups in each sample were quite stable after day 23 except group 6, *Lcc. lactis* and its relatives, appeared again after day 44.

4. DISCUSSION

4.1. The community structure and its dynamics

All 21 sequences had high similarity only to *Bacillaceae* (in Ribosomal Database Project[7], *Bacillus-Lactobacillus-Streptococcus* subdivision) and so they all could be classified into *Bacillaceae*, which includes genus *Bacillus*, *Lactococcus*, *Enterococcus*, *Staphylococcus*, *Macrococcus*, and so on. *Bacillaceae* is affiliated in Gram positive bacteria with low G+C content (GPBLGC). This result was consistent with that of the quinone profile

analysis (detailed data not shown). From day 2 on, major quinone molecules were MK-6 and MK-7, especially MK-7 after day 11[8]. Bacteria with MK-7 as a major respiratory quinone molecule are GPBLGC and *Cytophagales*[9], but *Cytophagales* were not observed by FISH (Fluorescent In Situ Hybridization) with probe specific to *Cytophagales*[8]. Thus, we could conclude that GPBLGC, and most probably *Bacillaceae*, was predominant after day 2.

In order to know the population dynamics at species/group level, we quantified the band intensity and describe the predominancy (Figure 6). We must be aware of several biases when using the result. First of all, nucleic acids are not evenly extracted from various bacterial species. Some species are known to be fastidious and difficult to extract with normal DNA extraction method. The second one is the PCR bias. The degree of multiplication by PCR method is different depend on DNA sequences. Moreover, location of the DGGE bands, or the degree of migration may affect the intensity of bands. However, we can still estimate the predominancy of bacterial species to some extent if we keep those limitation in mind.

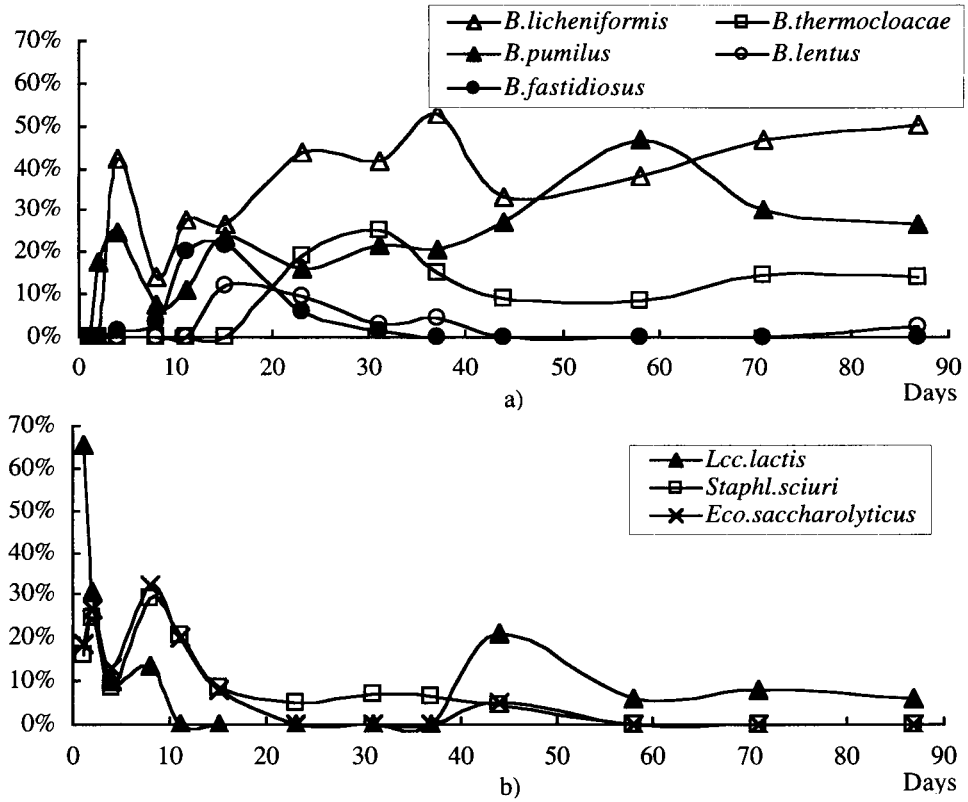

Figure 6. Predominancy of the bacterial groups calculated by the band intensity in eachsampling day. The percentage is the ratio to the total intensity of the visible bands in aDGGE lane. G1-G8 in the legends shows the group numbers in Table 1. a): genus *Bacillus*; b) other bacteria.

At the beginning of the operation, bacteria other than genus *Bacillus*, such as *Lactococcus*, *Staphylococcus* and *Enterococcus*, were predominated in the reactor. However, after day 4, *B. licheniformis* group became the most abundant. Other *Bacillus* group also appeared and *non-Bacillus* groups occupied the minor part. The composition of the groups did not change so much after day 23.

4.2. Process performance and community structure

The trends in temperature change can be separated into 4 periods. From day 0 to day 10, the thermophilic cycle started but an accident kept the reactor at low temperature later on. After the accident was recovered, periodical temperature cycles occurred except three batches until day 30. From day 30 to day 70, completely periodical temperature cycle was observed. The highest temperature in a batch decreased from day 70 and it did not rise from day 80 on. We call them as the initial period, the semi-stable period, the stable period, and the deteriorated period. This separation is in quite good agreement with the cluster analysis of the DGGE bands (Figure 5). Thus, the community structures were highly related to the temperature change. The relationship between the process performance and community structures were discussed in the four periods as follows.

During the initial period (from day 0 to day 10), some mesophilic fermentative bacteria like *Enterococcus*, *Lactococcus* and *Staphylococcus* were found. *Enterococcus* and *Lactococcus* can degrade starch and sucrose only fermentatively [10,11]. Thus, they fermented starch and sucrose in the substrate but contribute little to temperature rise owing to small energy generation in fermentation. On day 8, the DGGE band pattern was more similar to day 2 than day 4 (Figure 5). Those non-*Bacillus* mesophiles again became predominant (Figure 6). This result may have been linked to the decrease in reactor temperature between days 4 and 8 (Figure 2). In contrast, composition of the band intensity on day 4 was similar to that of day 11 and later. DGGE bands such as SP31-5, SP31-8 SP58-10, and SP31-14 appeared on day 4, disappeared on day 8 and reappeared after day 11. They were probably unknown obligate thermophiles although most of their closest relatives were mesophilic bacteria; *B.firmus*, *B.benzoevorans* and *B.cohnii*.

From day 10 to 30, the reactor performance was almost stable. The DGGE banding patterns showed that the community structure shifted to a stable state. The non-*Bacillus* mesophiles decreased and almost disappeared. Instead, obligate thermophilic *Bacillus*, *B. thermocloacae* appeared and stayed in the reactor until the end of the operation. *B. licheniformis*, facultative thermophilic *Bacillus*, got the predominancy in the community.

The performance of the reactor and the community structure was stable from day 30 to day 70. The community was composed primarily of *Bacilli*, including the close relatives of *B.thermocloacae* (obligate thermophile), *B.licheniformis* (facultative thermophile) and *B.pumilis* (mesophile). The group of *B.licheniformis* was the most abundant among them. Facultative thermophiles have an advantage under the conditions in this experiment because they can grow under both mesophilic and thermophilic conditions, which occurred in every batches. *B.licheniformis* can utilize starch and sucrose[12], and so they could grow in the reactor fed with those substrate. Moreover, some obligate thermophilic and mesophilic *Bacilli* also played some role on the reactor performance. They might be activated under suitable

temperature conditions and became dormant under adverse conditions. Generally speaking, Bacilli are tolerant under adverse conditions.

Even after the performance deteriorated and the temperature ceased to rise after feeding (day 80), the community structure seemed to remain constant according to DGGE. Thus, this deterioration was not caused by community change, but by some physical conditions. A probable reason is the moisture content of the media. It was 50 to 65% from day 10 to 60 but it rose above 70% after day 70. High moisture content reduces the diffusion rate of oxygen and hinders active aerobic metabolism. In composting process, it was reported that composting is impossible at the moisture content of 70% [13].

5. CONCLUSIONS

In the present study, microbial community structure in a TCOP reactor was determined by using PCR-DGGE and the sequences of the DGGE bands. The community structure and its dynamics could be related to the process performance comprehensively. Under high performance of TCOP, facultative thermophilic *Bacillus*, namely *B.licheniformis*, may predominate, but also obligate thermophilic *Bacillus* and mesophiles may play some role in the reactor. Based on the results, however, more quantitative methods such as FISH with genus/species specific probes or dot-blot hybridization must be employed to elucidate more precise composition of the communities to know better about reactor performance by community structures.

REFERENCES

1. Cai, H., and T. Mori. (1995) Proc. Environ. Eng. Res. Jpn. 32: 371-378.
2. Curtis, T., and N. Craine. (1997) p. 65-72. In Proc. of the Second International Conference on Microorganisms in Activated Sludge and Biofilm Process, IAWQ Specialist Group on Activated Sludge Population Dynamics, Berkeley, Calif.
3. Muyzer, G., E. C. Waal, and A. G. Uitterlinden. (1993) Appl. Environ. Microbiol. 59: 695-700.
4. Rolleke, S., G. Muyzer, C. Wawer, G. Wanner, and W. Lubitz. (1996) Appl. Environ. Microbiol. 62: 2059-2065.
5. Zhu, H., F. Qu, and L. H. Zhu. (1993) Nucleic Acids Res. 21: 5279-5280.
6. Hiraishi, A., Y. Morishima, and J. Takeuchi. (1991) J. Gen. Appl. Microbiol. 37: 57-70.
7. Maidak, B. L., G. J. Olsen, N. Larsen, R. Overbeek, M. J. McCaughey, and C. R. Woese. (1997) Nucleic Acids Res. 25: 109-111.
8. Kurisu, F., Satoh, H., Mino, T. and Matsuo, T. (1997) Proc. of Environ. Eng. Forum, Koriyama, Japan, 138-140 (in Japanese).
9. Collins, M. D., and D. Jones. (1981) Microbiol. Rev. 45: 316-354.
10. Farrow, J. A. E., J. Kruze, B. A. Phillips, A. J. Bramley, and M. D. Collins. (1984) System. Appl. Microbiol. 5: 467-482.

11. Sheleifer, K. H. , J. Kraus, C. Dvorak, R. Klilpper-Balz, M. D. Collins, and W. Fisher. (1985) System. Appl. Microbiol. 6: 183-195.
12. Claus D., and R. C. W. Berkeley. (1986) p. 1105-1138. In P. A. Sneath, N. S. Mair, M. E. Sharpe, and J. G. Holt (ed.), Bergy's manual of systematic bacteriology, 2nd ed., Vol. 2. The Williams & Wilkins Co., Baltimore, Md.
13. Bach, P. H., M. Shoda, and H. Kubota. (1984) J. Ferment. Technol. 62: 285-292.

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Relating function and community structure of complex microbial systems using neural networks

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Our ability to incorporate data that is obtained by using high throughput molecular techniques such as DNA based community fingerprinting, whole genome expression analysis, and protein spectrum analysis, into mathematical models is extremely limited. These techniques generate thousands of data points but only few and the most obvious relationships can be established, often only qualitatively. For the information to be useful for the control and management of a biological system, we must be able to quantify and establish the underlying relationships between community structure and function, preferably by mathematical tools. Currently, such mathematical tools do not exist since the underlying relationships between molecular data and function are too complex to be formulated. It is expected that approaches such as artificial neural networks (ANN) that do not require a priori knowledge of such relationships may be able to fill this gap. This study presents data on the application of ANN to link the function and community structure of two quadruplicate sets of laboratory-scale methanogenic microbial communities under shock load conditions. The community structure was characterized using terminal restriction fragment length polymorphisms, amplified ribosomal DNA restriction analysis, sequencing, and phase contrast and fluorescent microscopic techniques with image analysis. The results of this study indicate that ANN is a powerful tool to establish links between function and community structure provided sufficient data sets are available for training and testing of the network. In addition, it may also be useful to identify important microbial populations that correspond to various functions, which is not generally possible by other means. The limitations of neural networks to analyze data pertaining to complex microbial systems is also elucidated.

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1. INTRODUCTION

A number of techniques are now available for the characterization of microbial community structure (Table 1). They are generally based on the abundance of various types of nucleic acid, cell wall component, and morphological characteristics. Examples are: terminal restriction fragment length polymorphisms (T-RFLP; based on 16S rDNA gene fragment), amplified ribosomal DNA restriction analysis (ARDRA; based on clone abundance), microscopy with image analysis (based on morphotype quantification), and membrane hybridization of 16S rRNA. However, their use for predictive and control purposes through mechanistic mathematical models is non-existent and often impossible.

Table 1
Methods for community structure analysis

Method	Purpose	Advantages (+) and disadvantages (-)
1. Terminal restriction fragment length polymorphism	obtain 16S rDNA fragment fingerprint of a community	+: throughput, speed -: quantification, identification
2. Amplified ribosomal DNA restriction analysis	obtain 16S rDNA fingerprint of a community	+: identification after sequencing -: speed, throughput
3. Denaturing gradient gel electrophoresis	obtain DNA fingerprint of a community	+: throughput, speed -: quantification, identification
4. Microscopy/image analysis	obtain distribution of shapes and sizes of population(s)	+: biovolume quantification, speed -: identification
5. Reverse sample genome probing	obtain DNA fingerprint of target population(s) in a community	+: throughput, speed -: target populations must be known
6. Fatty acid methyl ester analysis	identity through cell wall composition	+: throughput, speed -: nonspecific changes in fatty acids
7. Flow cytometry with probes	track individual population(s)	+: numerical quantification, speed -: target population must be known, costly
8. Fluorescent in situ hybridization	track individual population(s)	+: spatial distribution, identification -: quantification, population must be known
9. 16S rRNA microarrays	obtain community fingerprint based on 16S rRNA phylogeny	+: throughput, speed, information -: 16S rRNA targets must be known, costly, developing
10. Protein spectrum analysis	obtain protein spectrum of a community or population	+: throughput, speed, information -: costly, developing

This is due to the lack of knowledge about the underlying relationships between these measurements and the community function and also due to the associated objections with many of these methodologies, e.g., biases in DNA extraction, cloning, and polymerase chain reaction (PCR), and variation in ribosomal copy number etc. Molecular data and mechanistic models are simply not compatible until the underlying relationships between function and community structure as described by these methods are known. The need for improving the mechanistic models, however, is obvious since the application of ecological principles will most likely require models with a resolution finer than the total biomass. For example, mathematical models for improved control of an anaerobic digestor must consider different guilds and numerically non-dominant but important syntrophic populations separately. Similarly, models representing bulking in activated sludge systems must describe the dynamics of filamentous bacterial populations causing sludge bulking. Since the existing mechanistic models are not able to utilize community structure data efficiently, it is imperative to explore other modeling techniques that are more compatible with molecular data.

This paper presents artificial neural networks (ANN) as a potential tool for modeling of microbial communities when community structure data is obtained through molecular techniques and application of mechanistic models is not feasible. ANN does not require mechanistic assumptions about the underlying relationships. Hence, it is especially suited to link molecular data with function. It learns the underlying relationships by examples of data sets presented to it and has been used in many different fields. In addition, the output of ANN can also be used for online control of biological processes. Various applications of ANN can be classified into the following four classes.

(a) Classification/interpretation, i.e., inverse mapping from observation to known classes: this application is similar to principal component analysis and cluster analysis. It is the most common application of ANN. Examples pertinent to biological processes include identification of rep-PCR genomic fingerprints [1], tracking soil microbial communities by denaturing gradient gel electrophoresis [2] and phospholipid analysis [3], development of an automated systems for the identification of heterotrophic marine bacteria on the basis of their fatty acids composition [4], restriction pattern recognition of *Escherichia coli* 0157:H7 [5], and cluster analysis of whole genome expression data [6].

(b) Diagnosis, i.e., inverse mapping from observed effect to cause: identifying disease from symptoms.

(c) Control, i.e., inverse mapping from observed state to control forces: An example in this category is the use of neural network modeling in optimization of continuous fermentation processes [7].

(d) Prediction, i.e., future course of a system based on past patterns: Examples include an assessment of neural networks and statistical approaches for prediction of *E. coli* promoter sites [8], and predicting grassland community changes with an artificial neural network model [9].

In addition, various applications in ecosystem modeling are also reported [10-13].

2. MATERIALS AND METHODS

2.1 Bioreactor operation and perturbation:

Two quadruplicate sets of variable volume anaerobic reactors were operated for 80 days and 32 days, respectively at an average glucose loading rate of 0.34 g/L-d and an average mean cell residence time (MCRT) of 16 days as described previously [14]. Based on the numerically most dominant population, they are referred to as low spirochete set (LS set; reactors 1-4) and high spirochete set (HS set; reactors 5-8), respectively. Inocula for both sets were obtained from two 18-L completely mixed stirred tank "mother" reactors originally seeded from the local anaerobic digester sludge and operating at 0.5 g/L-d glucose loading rate and at MCRT of 16 days. The mother reactor for the HS set was operated for more than 4 years under two different conditions: for the first 3 years it was operated at a glucose loading rate of 0.8 g/L-d and an MCRT of 10 days. Nearly 200 days prior to the inoculation of the HS set, this reactor received fresh digester sludge and was switched to a 0.5 g/L-d organic loading rate and an MCRT of 16 days. The mother for the LS set was operated only for two months at an MCRT of 16 days and at an organic loading rate of 0.5 g/L-d. At the time of seeding the two sets, both mother reactors were operating at more than 90% chemical oxygen demand (COD) removal efficiency. All daughter reactors were maintained in 500 mL sealed reagent bottles with liquid volume cycling between 250 mL to 362 mL over a period of eight days. All reactors were maintained at 35 °C in temperature controlled water baths under mixed conditions. A glucose perturbation was applied by increasing the reactor concentration to 6.8 g/L using a stock solution of glucose that also contained equivalent amounts of bicarbonate solution. Samples were collected at appropriate intervals after the perturbation for functional and community structure analysis.

2.2 Functional analysis

The overall efficiency of removal was monitored by effluent COD measurement and soluble products analysis. Suspended solids were removed by centrifugation at 6000 g for 15 minutes and an appropriate amount of the supernatant was digested for COD analysis using Hach 0-1500 ppm range COD vials (Hach Company). Glucose and soluble products were analyzed using high performance liquid chromatography equipped with a 300-mm HPX-87H ion exclusion column (Bio-Rad Laboratories) connected to a UV/Vis absorbance detector (Shimadzu) set at 210 nm and a refractive index detector (Waters, Inc.). The mobile phase used was 0.013 N H_2SO_4 at 0.7 mL/min with the column temperature set at 65 °C. Supernatant sample after centrifugation was filtered through 0.22 μm filter (Whatman, Inc.), acidified with 0.1 M H_2SO_4, and injected (100 μL) onto the HPLC. Volatile fatty acids were identified by retention time comparison of authentic material on the HPX-87H column and also by GC/FID analysis using a 4 mm i.d. and 183 cm long glass column (GP10%, SP-1200/1% H_3PO_4) and a Hewlett Packard 5890A series II gas chromatograph. Headspace methane was analyzed using gas chromatography (Hewlett-Packard, Model 5890). Hydrogen accumulation in the headspace was measured using a Trace Analytical RGA3 reduction gas analyzer. The source and purity of all chemicals used in this study for feeding or standardization were same as described previously [14].

2.3. Community structure analysis

Reactor samples collected before and during the perturbation were subjected to total biomass analysis, ARDRA, T-RFLP, and image analysis. Only the later two techniques are

briefly reproduced here. For other techniques and further information please refer to sources described elsewhere [15].

T-RFLP analysis was done on extracted DNA from the reactor samples using a protocol described previously [16]. Briefly, 16S rDNA was amplified from extracted genomic DNA using PCR and universal primers (8F and 1392R, *E. coli* numbering) of which the forward primer was labeled with HEX dye. The amplified and labeled products were digested with three restriction enzymes (MspI , RsaI, and HhaI) separately and the resulting digested fragments were visualized using an ABI gene sequencer in Gene Scan mode.

Morphological quantification was done by microscopy and image analysis of freshly collected bioreactor samples as described previously [15]. Microscopy was done using a Zeiss Photomicroscope I equipped with a 100X PlanApochromat phase 3 oil immersion objective. Classification of morphological units was performed using Bioquant System IV and MegX image analysis software. Biovolumes were calculated after determining the mean volume per cell for each different morphotype using the CMEIAS object analysis plug-in operating in UTHSCSA ImageTool Ver. 1.27 software. The data for this study were derived from image analysis of 1,440 photomicrograph negatives (averages of 1,145 cells per reactor sample).

2.4. Artificial neural network analysis

The artificial neural network analysis was performed with three hidden layers, full connections, and back propagation algorithm using NeuroSolutions [17] both for the example data sets for ΔG calculation and also for T-RFLP and image analysis. The number of nodes in the input and output layer corresponded with the number of parameters (fragment lengths, morphotype or accumulated intermediates) in the input and output data. The community structure data served as the input and function data served as the desired output. For relating T-RFLP data to function, fragment lengths from all three restriction enzymes were pooled together for the analysis. Also, due to PCR bias, only the presence or absence of a fragment was considered for T-RFLP data with no emphasis on the abundance of each fragment. The data from all the eight rectors and up to five time points for each reactor was divided into two groups: 80% for training the network and 20% for testing the predictive capability of the trained network. Once the training was completed and the error for the prediction set was minimal, sensitivity analysis was carried out to find the dependence of the network in mapping each input parameter to the output parameter.

3. RESULTS AND DISCUSSION

3.1. An example of sensitivity analysis

Figure 1 shows the results obtained from a simple application of ANN by selecting an example (ΔG calculation of propionate conversion to acetate) where a mechanistic model is also available. Ordinarily, ANN will not be used in such scenarios. The example is presented here in order to show that the sensitivity analysis of a trained ANN can indicate the importance of each input parameter in determining the output. The sensitivity analysis is relevant in linking the function to community structure.

The equation that is used to calculate the free energy available (ΔG) during the conversion of propionate to acetate is given by:

$$\Delta G = \Delta G^{0'} + RT \ln \frac{[CH_3COO^-][H_2]^3[CO_2]}{[CH_3CH_2COO^-][H_2O]^2} \qquad (1)$$

where, values in brackets on the right hand side of the equation represent concentration in moles for aqueous or atm for gaseous species, R is universal gas constant, T is temp in °K, and $\Delta G^{0'}$ is the free energy of reaction under standard conditions .

The same value of ΔG can also be obtained using ANN if sufficient examples are presented for training. Thus a mechanistic model requires the equation itself while ANN requires sufficient number of input and output data sets. Results presented in Figure 1b demonstrate that the network is more sensitive to parameters such as H_2 and less sensitive to those parameters that do not affect the outcome. It should also be noted that for an unknown system, selection of the input parameter is a difficult task because ANN will not be able to decide *a priori* if an input parameter is irrelevant for obtaining the output. Given sufficient number of examples, it can, however, show the insensitivity to those input parameters that change but do not affect the output.

3.2. Application of sensitivity analysis to community structure and function

Functional response (given by soluble products analysis) and community structure data (given by T-RFLP and microscopy/image analysis) in response to the perturbation for one out of eight reactors (reactor 7) is shown in Figure 2. It is obvious that even if the fragment

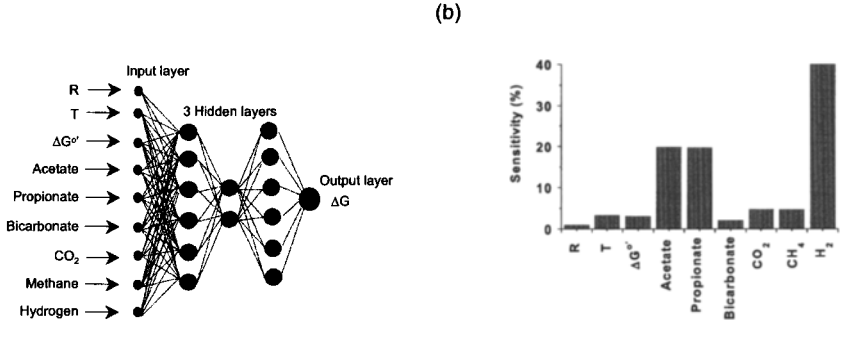

Figure 1. (a) The architecture of neural network for ΔG calculation, (b) sensitivity of the network to various input parameters.

identities are known, this community structure analysis will not yield to mechanistic mathematical modeling because of the lack of knowledge between the presence of a 16S rDNA fragment, morphotype, and the corresponding function. It is also apparent that methods in addition to community analysis are needed to establish links between the community characterization techniques and function. Both cluster analysis and sensitivity analysis of the two data sets was performed. Since the sensitivity analysis is more relevant with respect to providing links between function and structure, only the later results are presented here.

Figure 3 shows the sensitivity analysis after training a network similar to shown in Figure 1 using T-RFLP and functional data. Input layer in this case received information about the presence or absence of a given ribotype represented by 1 and 0, respectively. As seen in Figure 3, some ribotypes have greater sensitivity than others indicating that their presence or absence is playing a bigger role in the accumulation of the corresponding soluble intermediate. Whether this indication of a correlation with function is significant can only be determined after further experiments focusing on the identification of the fragments and isolation of the organisms. ANN does provide with new hypothesis e.g., is ribotype 527 bp (Figure 3a) belongs to a butyrate oxidizer?

A similar analysis performed with the image analysis data representing the morphotypes is shown in Figure 3c. It is understood that a single morphotype most likely contains a large number of species, similar to the T-RFLP analysis where a single ribotype is not necessarily a single organism [10]. However, increase in a given morphotype is indicative of the presence of an organism that belongs to the morphotype and responded to the perturbation. As seen from Figure 3c, different groups of morphotypes correlated with different soluble products. For example, straight rods in chains and spirals were equally sensitive to all three soluble intermediate products, but thin filaments and large thick rods correlated strongly with butyrate only.

T-RFLP and image analysis are among the fastest ways to study the dynamics of mixed microbial communities (hundreds of samples can be analyzed in a week), easy to automate, and reproducible (Figure 3b). ANN improves the usefulness of these techniques by providing a way to link these measurements with the community function. Several other reasons may also be advanced for using ANN for complex microbial communities. ANN has the potential to model chaotic (irregular in appearance and deterministic in underlying rules [18]) and statistical (regular and probabilistic) phenomena. Since mixed microbial communities are closer to the above types of phenomena as opposed to regular (regular and deterministic) or random (irregular and probabilistic) phenomena, ANN could serve as a powerful tool to describe them. In addition, the gap between our ability to collect community characterization data and link it with function will continue to grow as the use of high throughput techniques e.g., microarrays and protein spectrum analysis find their way into mixed community management. With no tools available immediately to determine the underlying relationships, it may be useful to test ANN for finding new relationships and generate hypothesis. It may also be useful as a tool to develop new mechanistic models for complex biological systems.

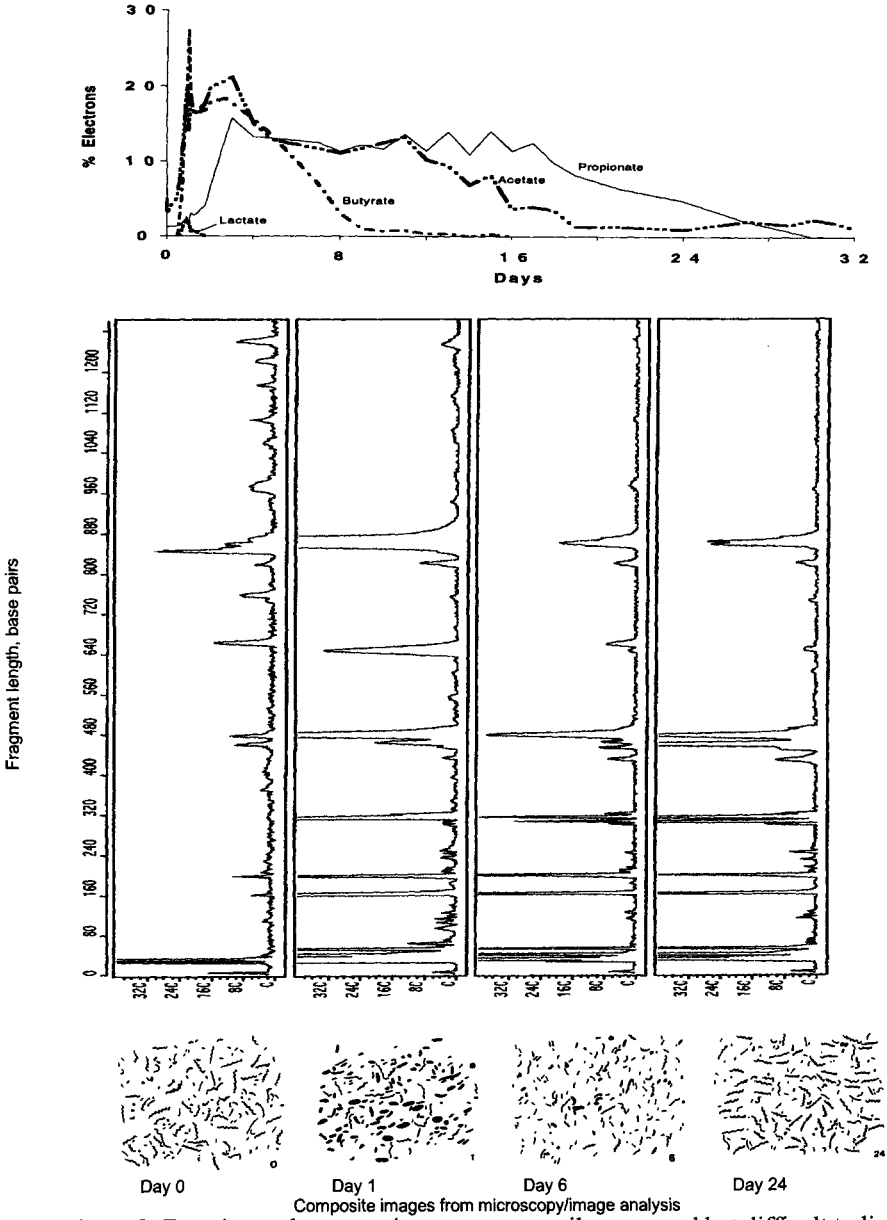

Figure 2. Function and community structure - easily measured but difficult to link

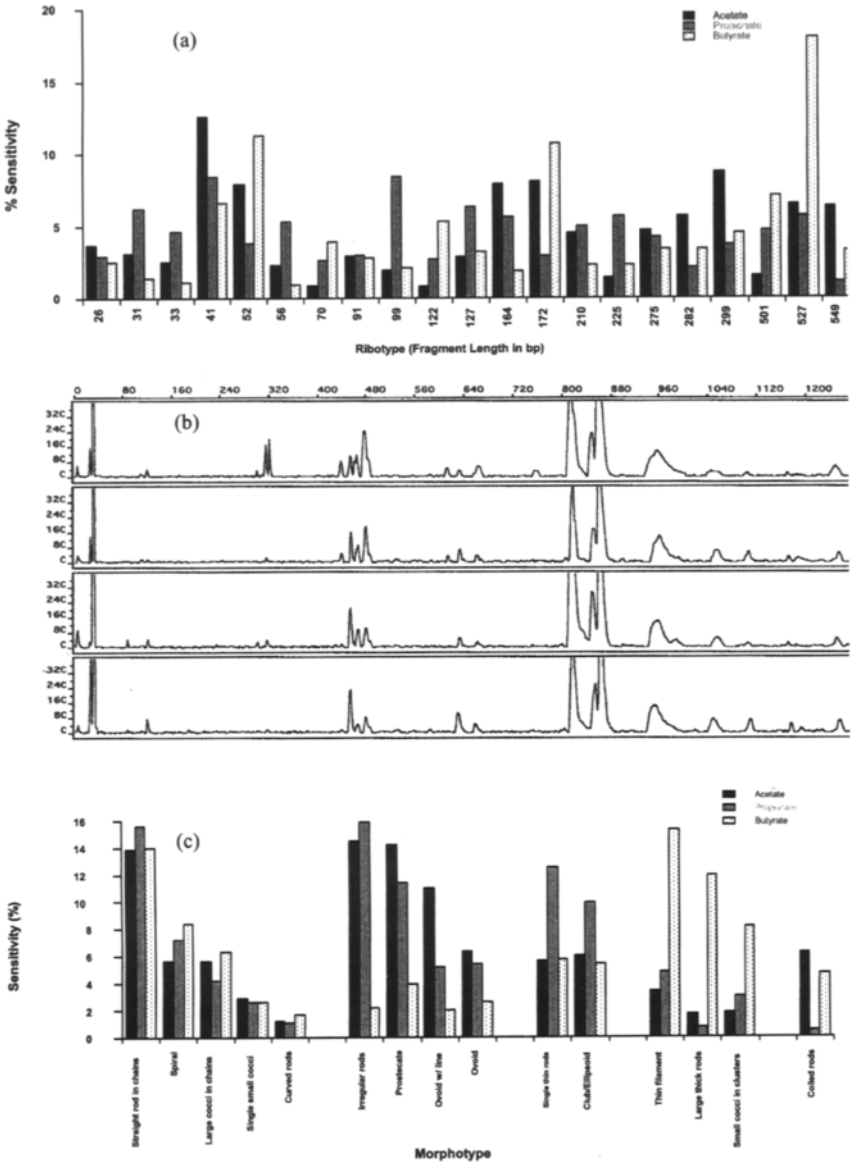

Figure 3. Sensitivity analysis for ribotype (a), replication of community structure before the perturbation in reactors 5-8 as given by RsaI generated ribotypes (b), and sensitivity analysis for morphotype using microscopy/image analysis (c).

The limitations of ANN are as important as its power to relate two data sets without the knowledge of the underlying relationships. It must be pointed out that the existence of a relationship between the data sets presented to the network is the responsibility of the user. The number of example data sets to train the network and test the training quality for predictive purposes must be large. Also, the network must be properly trained. Over trained networks are equivalent to memorization resulting in poor predictive power. Lastly the predictive power of ANN is based on history, not wisdom. Hence, prediction about completely unseen scenarios should be avoided.

4. CONCLUSIONS

Artificial neural networks are more suitable modeling tools for molecular data than mechanistic approaches. They do not require the underlying rules and therefore utilize the data more efficiently. ANN may also be helpful in determining the importance of a community member (ribotype for T-RFLP and morphotype for image analysis) with respect to a functional or environmental parameter.

REFERENCES

1. Tuang, F.N., J. L.W. Rademaker, E.C. Alocilja, F.J. Louws, and F.J. de Bruijn. 1999. *FEMS Microbiology Letters.* 177(2): 249-256.
2. Almeida, J.S., K. Leung, S.J. Macnaughton, C. Flemming, M. Wimpee, G. Davis, and D.C. White. 1998. *Bioremediation.* 1:255-264.
3. Noble, P.A., J.S. Almeida, and C.R. Lovell. 2000. *Applied and Environmental Microbiology.* 66(2):694-699.
4. Bertone, S., M. Giacomini, C. Ruggiero, C. Piccarolo, and L. Calegari, 1996. *Applied and Environmental Microbiology.* 62:2122-2132.
5. Carson, C.A., J.M. Keller, K.K. Mcadoo, D.Y. Wang, B. Higgins, C.W. Bailey, J.G. Thorne, B.J. Payne, M. Skala, and A.W. Hahn, 1995. *Journal of Clinical Microbiology.* 33: 2894-2898.
6. Partek Incorporated. 2000. http://www.partek.com/.
7. Lednickey, P., and A. Meszaros. 1998. *Bioprocess Engineering.* 18(6): 427-432.
8. Horton, P.B., and M. Kanehisa. 1992. *Nucleic Acids Research.* 20:4331-4338.
9. Tan Sen, S., and F. Smeins. 1996. *Ecological Modelling.* 84(1-3). 91-97.
10. Foody, G.M. 1999. *Ecological Modelling.* 120: 97-107.
11. Gross, L., S. Thiria, and R. Frouin. 1999. *Ecological Modelling.* 120: 237-246.
12. Moatar, F., F. Fessant, and A. Poirel. 1999. *Ecological Modelling.* 120: 141-156.
13. Schleiter, I.M., D. Borchardt, R. Wagner, T. Dapper, K. Schmidt, H. Schmidt, and H. Werner. 1999. *Ecological Modelling.* 120: 271-286.
14. Hashsham, S.A., A.S. Fernandez, S.L. Dollhopf, F.B. Dazzo, R.F. Hickey, J.M. Tiedje, and C.S. Criddle. 2000. Parallel processing of substrate correlates with greater functional stability in methanogenic bioreactor communities perturbed by glucose. *In press.*
15. Fernandez, A.S., S.A. Hashsham, S.L. Dollhopf, L. Raskin, O. Glagoleva, F.B. Dazzo, R.F. Hickey, C.S. Criddle, and J.M. Tiedje. 2000. Flexible community structure correlates with stable community function in methanogenic bioreactor communities perturbed by glucose. *In press.*

16. Liu, W.T., T.L. Marsh, H. Cheng, and L.J. Forney. 1997. *Applied and Environmental Microbiology*. 63(11):4516-4522.
17. NeuroDimension, Inc. 1999. http://www.nd.com/.
18. Toshinori, M. 1998. Fundamentals of the new artificial intelligence: beyond traditional paradigms, Springer-Verlag.

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Comparison of microbial communities in anaerobic granulated sludge reactors treating benzoate, methyl benzoate and terephthalate

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Microbial community of anaerobic granulated sludge treating benzoate (BA), methyl benzoate (MBA) and terephthalate (TA) were compared. Although BA, MBA and TA differed from one another only in the functional group (i.e., H-, COOH-, and CH_3-), the community fingerprint and composition as determined by denaturing gradient gel electrophoresis (DGGE) of the amplified community 16S rDNA and clone library of 16S rDNA statistically showed a significant level of difference. The DGGE fingerprint of TA-degrading consortium was more closely related to MBA- than to BA-degrading consortium. Clone library revealed that the predominant archaeon in all consortia was *Methanoseata*. Bacterial populations were affiliated mainly with the γ- and δ-subdivisions of the division *Proteobacteria*, followed by at least 7-9 proposed bacterial divisions (P. Hugenholtz, R. Goebel and N. R. Pace, J. Bacteriol. 180:4765-4774, 1998). Furthermore, 16S rRNA sequences in the δ-*Proteobacteria* were very diverse and closely affiliated with known syntrophic bacteria and sulfate reducers, or formed novel phylogenic groups within the δ-*Proteobacteria*. The phylogenic diversity of those presumptively syntrophic H_2-producing acetogens was significantly greater than currently known syntrophic bacterial isolates, and warrants further study.

1. INTRODUCTION

Anaerobic biological treatment systems (e.g., upflow anaerobic sludge blanket [UASB] and anaerobic fluidized bed [AFB] reactors) represent a proven sustainable technology for treating a wide range of very different industrial waste streams in Taiwan (>10 million M^3 per year). The technology relies on the formation of balanced or syntrophic microbial communities in either suspended (i.e., granulation) or immobilized (i.e., biofilter) forms. In these anaerobic microbial consortia, degradation of complex pollutants usually involves three phases: hydrolysis/acidogensis, acetogenesis, and methanogenesis [1]. During these phases, complex pollutants are first hydrolyzed and acidified by acidogenic bacteria forming volatile fatty acids,

which are then converted to acetate and CO_2/H_2 by H_2-producing acetogenic bacteria. Finally, these intermediates are further converted to methane by methanogenic bacteria.

The current knowledge on the optimization and process control of those anaerobic treatment systems for treating toxic/inhibitory compounds is still limited. It was until recently that studies have shown the ability of UASB systems to degrade aromatic compounds including benzoate [2], phenol [3,4], cresol [5], terephthalate [6-12], methyl-benzoate [9,13], and nitro- or azo-substituted aromatics [14]. The slow progress on the study was because that a long acclimation time or start-up period was required to establish proper anaerobic consortia that degrade aromatic compounds [3,4,6,11,13]. The length of start-up period varied from a few months to more than 600 days. This long start-up period was possibly due to (1) the toxicity of those aromatics on the anaerobic microbial consortia, and (2) the absence or scarcity of microbial populations that can degrade those aromatics in the seed sludge. The first possibility was supported by the study of Reyes *et al.* [15] that showed the structure-toxicity relationships of different aromatic compounds to acetoclastic methanogenic bacteria. Based on the lag phase length and the gas production rate, they indicated that the type of functional groups had a profound effect on the toxicity of the test compound. The degree of toxicity for those functional groups in an increasing order was COOH-, SO_3H-, H-, OH-, CH_3-, CHO-, COH_3- and Cl-. By reducing the organic loading, the operation of a lab-scale UASB process degrading methyl benzoate was succeeded [13]. However, the second possibility remained unverified because very little was known about the anaerobic microbial consortia degrading various aromatics.

Poor understanding on the microbial diversity of aromatics-degrading anaerobic consortia in UASB processes was in part limited by the techniques previously available. In the past, cultivation technique was often used to isolate and identify microorganisms involved in the degradation of aromatics [16,17], but was shown to be very difficult for isolating anaerobic syntrophic bacteria [18]. Other studies have used electron microscope to identify microorganisms based on morphology, but often failed to differentiate closely related bacteria [2,19,20].

It is becoming apparent that molecular technique is a potential tool to study microbial organization and its function in complex ecosystems [21-25]. These molecular techniques have been successfully applied to identify syntrophic propionate-oxidizing bacteria [26], sucrose/propionate/acetate-utilizing populations [27] and trichlorobenzene-transforming populations [28], and to localize the methanogens [28,29] in the anaerobic granular sludge systems. This communication further describes the use of molecular approaches to better define the constellation of microbial consortia involving in the degradation of three different aromatic compounds including benzoate (BA), terephthalate (TA), and methylbenzoate (MBA) in UASB systems. In addition, the effect of chemical types and seeding sludge sources on the microbial community was evaluated.

2. MICROBIAL DIVERSITY OF UASB SYTEMS DEGRADING AROMATIC COMPOUNDS

2.1. Methanogenic granular consortia

In the absence of exogenous electron acceptors (e.g., oxygen, nitrate, and sulfate), the first step of degradation of aromatic compounds in UASB systems is an acetogenic process that involves several microbial populations. The first microbial population(s) would need to

convert the aromatic compounds like benzoate to acetate and hydrogen. However, as Equation (1) indicates the conversion of benzoate to acetate and H_2 was thermodynamic unfeasible under standard conditions (pH 7.0, 1 M concentration, 1 atm and 25°C). Thus, a coupling reaction for example that converts acetate or HCO_3^-/H_2 to methane and CO_2 as shown in Equations (2) and (3) is needed to lower the Gibbs free energy. Therefore, in this communication, the microbial consortium that degrades aromatics could be referred as a syntrophic methanogenic consortium, and the acetogenic bacteria or the fermentative bacteria transforming aromatic compounds to acetate and hydrogen were referred as syntrophic H_2-producing acetogens.

Equation 1: Benzoate- + $7H_2O \rightarrow$ 3 acetate$^-$ + 3 H^+ + HCO_3- + $3H_2$ $\Delta G^{o\prime}$= 53 kJ

Equation 2: Acetate- + $H_2O \rightarrow CH_4 + HCO_3^-$ $\Delta G^{o\prime}$= -31 kJ

Equation 3: $HCO_3^- + 4H_2 + H^+ \rightarrow CH_4 + H_2O$ $\Delta G^{o\prime}$= -135.6 kJ

Sludge granulation by the syntrophic methanogenic consortium is another key feature observed in UASB systems. These microbial populations usually aggregate in a close spatial orientation or juxtaposition to facilitate the interspecies transfer of intermediates, H_2 and CO_2 among acidogens, acetogens and methanogens [31,32]. The structure of the granule could be further classified as layered and uniform types, and were found to be highly dependent on the degradation kinetics of a substrate (e.g., one, two or three step processes) [33].

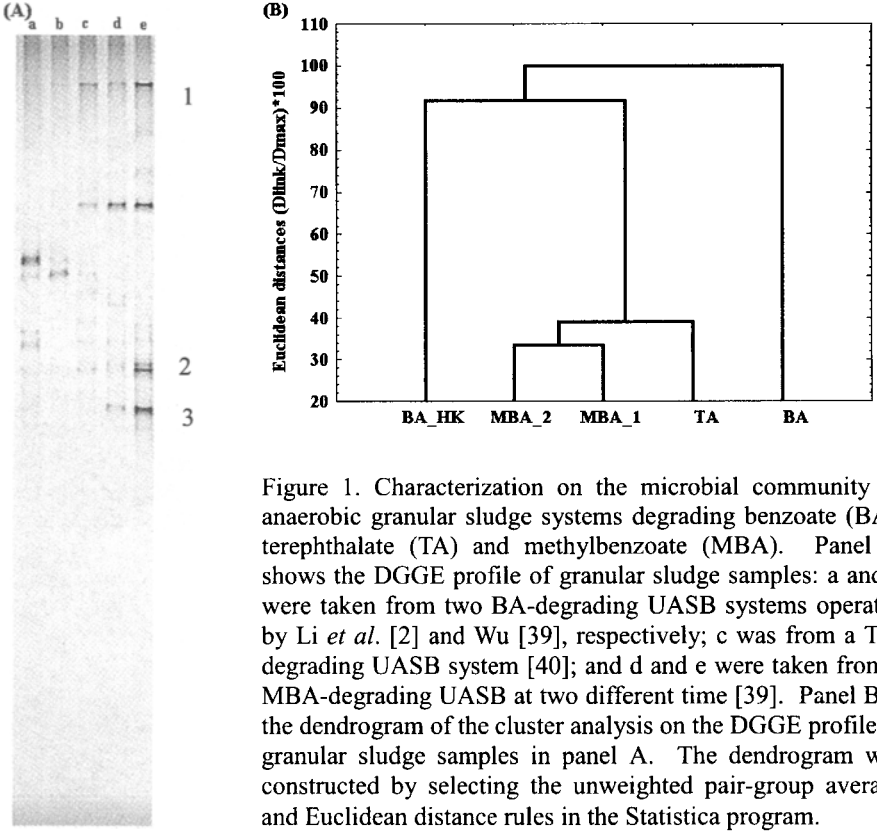

Figure 1. Characterization on the microbial community of anaerobic granular sludge systems degrading benzoate (BA), terephthalate (TA) and methylbenzoate (MBA). Panel A shows the DGGE profile of granular sludge samples: a and b were taken from two BA-degrading UASB systems operated by Li *et al.* [2] and Wu [39], respectively; c was from a TA-degrading UASB system [40]; and d and e were taken from a MBA-degrading UASB at two different time [39]. Panel B is the dendrogram of the cluster analysis on the DGGE profile of granular sludge samples in panel A. The dendrogram was constructed by selecting the unweighted pair-group average and Euclidean distance rules in the Statistica program.

2.2. Microbial community structure as revealed using DGGE

For initial studies of the microbial diversity and similarity of different aromatics-degrading UASB systems, we used the denaturing gradient gel electrophoresis (DGGE) method [34]. Total community DNA of sludge samples were recovered using techniques that are well established by our group [24]. The DGGE analyses with domain-level PCR primers [35,36] was performed as previously described [37]. Figure 1A shows the DGGE analysis of the bacterial 16S rDNA gene fragment amplified from the granular sludge. Samples a and b were taken from two BA-degrading UASB systems that differed in seed sludge. Sample c, and samples d/e were taken from a TA- and a MBA-degrading UASB system, respectively. Samples d and e were taken from the same reactor at about 10 months apart. Those systems where samples b-e were taken were all enriched with the same seed sludge. Based on the DGGE banding pattern, approximately 17 detectable different fragments were observed among those five samples, but none of these fragments was common to all samples. Furthermore, samples c, d and e appeared to contain more detectable fragments than samples a and b.

The similarity of microbial community structure among those five samples was statistically analyzed (Fig. 1B). Between the two BA-degrading samples (BA and BA_HK, Fig. 1B), the DGGE banding pattern for the bacterial population was not identical, suggesting that different seeding sludge could have led to the selection of different BA-degrading populations in the microbial consortia. The DGGE banding patterns among samples BA, TA, MBA_1, and MBA_2 were also different. This result further suggests that different aromatic compounds could have led to the selection of different microbial populations in UASB systems. Still, the DGGE banding pattern of sample TA was relatively more similar to those of samples MBA_1 and MBA_2 than to samples BA_HK and BA. Samples MBA_1 and MBA_2 taken from a same reactor but at different time exhibited a similar DGGE banding pattern. Increase in the band intensity of fragments 1-3 in sample e (Fig. 1A) suggested that prolonged enrichment has promoted the dominance of these populations in the process. Based on these results, it could be concluded that seed sludge source and aromatic compounds could affect the formation of microbial community structure selected in the UASB systems.

2.3. Phylogenic diversity as revealed by 16S rRNA sequence analysis

Our recent studies using the 16S rDNA clone library approach have further provided a preliminary and the first insight into the phylogenic diversity of microbial populations in UASB systems degrading benzoate (BA), terephthalate (TA), and methylbenzoate [38-40]. Phylogenic analysis on the 16S rRNA sequences revealed that major archaeal 16S rRNA sequeces in BA- and TA-degrading granules were all close relatives of methanogens in the division *Euryarchaeota*. Majority of these sequences were closely affiliated with *Methanosaeta concilii* (formerly known as *Methanothrix soehngenii*) (99% sequence similarity) that convert acetate to methane [41]. The *Methanosaeta* sp. was also suggested to be important to sludge granulation [42,43]. Other archaeal sequences found were closely related to the genera *Methanospirillum* and *Methanogenium* in the family *Methanomicrobiales*. These findings closely allied with previous studies that both the acetoclastic *Methanosaeta* sp. and the hydrogenotrophic methanogen (i.e., *Methanospirillum, Methanobacterium and Methanobrevibacter*) were frequently found in anaerobic sludge granules [19,42,43].

Table 1 shows a high phylogenic diversity among those syntrophic H_2-producing acetogens found in the BA-, TA- and MBA-degrading UASB systems. At present, the domain *Bacteria* is classified into at least 36 divisions or kingdoms [44]. According to this classification, the predominant phylogenetic groups found in those three consortia were mainly affiliated with the γ- and δ-subdivisions of the *Proteobacteria.* In addition, 16S rRNA sequences were found to affiliate with seven other known divisions (*Cytophagales*, the low G+C Gram-positive group, Spirochetes, green non-sulfur bacteria [GNS], *Synergistes*, OP8 and OP10). Furthermore, six other 16S rRNA sequences were found to have a low degree of similarity to known sequences, and some of them could possibly be assigned to a novel or candidate division (i.e., sequence <80% identical to known sequences [44]). The common bacterial divisions found among the BA-, TA-, and MBA-degrading systems were GNS and the δ-*Proteobacteria*. Interestingly, most of these diversified phylogenic populations were also found in the microbial community of an AFB treating trichlorobenzene (TCB). In addition to those phylogenic divisions found in UASB systems degrading BA, TA and MBA, the α- and β-subdivisions of the *Proteobacteria*, *Acidobacteria*, green sulfur bacteria, and divisions TM6, OP10 and WS1 were reported.

Figure 2 further reveals the phylogenic diversity of predominant 16S rRNA sequences (20 in total) found within the δ-subdivision of the *Proteobacteria*. Most of these sequences were closely associated with genera that are known either to form syntrophic relation (i.e., *Syntrophobacter*, *Syntrophus*, and *Smithella propionica*) with methanogens, or to perform sulfate reduction (e.g. *Desufovibrio baarsii* and *Desulfosarcina variabilis*). Other sequences were affiliated to novel δ-groups (i.e., clone OPT23, clone OPB16, and clone SJA) or could be classified into five novel groups(i.e., clone MBA_9, MBA_25, MBA_44/56, TA_C5, and TA_D6). Especially, the novel group TA_D6 was consisted of seven different but closely related sequences retrieved from the TA-degrading UASB system and two other sequences previously reported [28-46]. These results together with those in Table 1 and Fig. 1 further suggest that a difference in the predominant phylogenic populations among those UASB systems degrading BA, TA and MBA)

Clearly, the phylogenic diversity of presumptively syntrophic acetogen in anaerobic systems degrading aromatic compounds was high and significantly greater than that revealed by the cultivation technique. At present, the known syntrophic H_2-producing acetogenic bacterial isolates were classified into at least five different genera in the δ-subdivision of the division *Proteobacteria* and the low G+C Gram-positive group [17,47,48]. They were found mainly in anaerobic digestors, and a few of them were isolated from marine and freshwater sediments and rumen [17,47,48]. Among these syntrophic acetogens, only two isolates were reported to utilize aromatic compounds when grown in co-culture with methanogenic bacteria [16,17], suggesting a very limited understanding on the aromatics-utilizing syntrophic aceogens existing in natural and engineered ecosystems.

Overall, our preliminary studies [38-40] successfully demonstrated the capability of the molecular approach to characterize the phylogenic diversity of methanognenic granular systems degrading various aromatic compounds. The DGGE analysis indicated that the type of aromatic compounds and the source of seed sludge could lead to the selection of different microbial populations in the methanogenic consortia. Apparently, the phylogenetic diversity of syntrophic acetogens has exceeded our current understanding. Since the phylogenetic placement of 16S rRNA sequence information could not correctly reflect the metabolic and functional traits of bacterial populations, the exact metabolic traits of those novel populations warrants further studies.

Table 1
A molecular measure on the phylogenic diversity of bacterial populations in anaerobic biological treatment systems degrading aromactic compounds.

Habitats and substrate type	Total 16S rRNA sequences[a]	Bacterial division																														References
		Proteobacteria					*Cytophagales*	*Actinobacteria*	Low-G+C Gram +	*Acidobacterium*	*Verrucomicrobia*	Spirochetes	Nitrospira	GNS	OP11	Planctomycetes	Green sulfur	TM7	TM6	*Thermus/Deinococcus*	*Cyanobacteria*	*Synergistes*	OP8	Termite group I	OS-K	*Chlamydia*	OP3	OP10	WS1	OP5	Marine group A	*Fibrobcacter*	*Flexistipes*	*Dictyoglomus*	*Thermotogales*	*Thermodesulfobacterium*	*Aquificales*	others	
		α	β	γ	δ	ε																																
AnFB_TCB	51	3	8	1	6			2	8	3		3		11			1		1									1									28
UASB_BA	19			10	3				1					2								3														38
UASB_TA	15				10									1								1													3	40
UASB_MBA	19			2	7		2		1			1		2									1													3	39

a, # of unique 16S rRNA sequences as determined by restriction enzyme digestion, denaturing gradient gel electrophoresis, or direct sequencing.
AnFB, anaerobic fluidized bed reactor; UASB, upflow anaerobic sludge bed; TCB, trichlorobenzene; BA, benzoate; TA, terephthalate; and

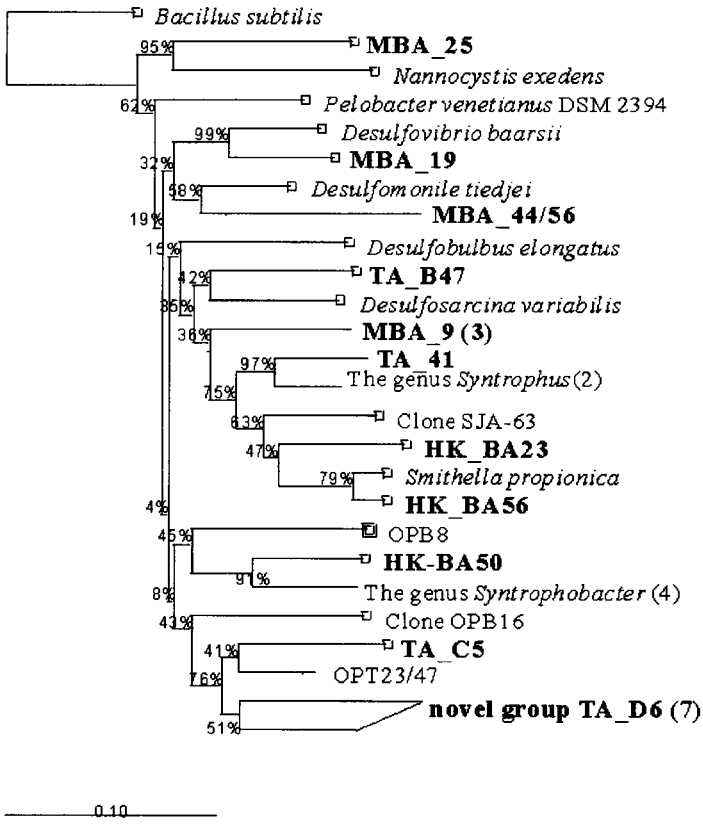

Figure 2. Phylogenic analysis of numerically dominant 16S rRNA sequences that were affiliated with the delta subdivision of the Proteobacteria in the clone library constructed for the BA-degrading UASB system operated at Hong Kong [2,38], and the TA- and MBA-degrading UASB systems [39,40]. The distance matrix consensus tree was calculated using the neighbor-joining method with bootstrapping in a sequence editor program, ARB [49]. The 16S rRNA sequence of *Bacillus subtilis* (belonging to the Gram-positive low G+C group) was used to root the tree. Bootstrap probabilities were indicated at the branch points (100 bootstrap data sets were used). Number in parenthesis indicates the total number of 16S rDNA sequences. Bar = 10 nucleotide substitutions per 100 nucleotides in 16S rDNA sequences.

REFERENCES

1. G. Letinga and L.W. Hulshoff Pol, Wat. Sci. Tech. 24(8) (1991) 87.
2. Y.Y. Li, H.H.P. Fang, T.Chen and H.K. Chui, J. Environ. Eng, 121 (1995) 748.
3. H.H.P. Fang, T. Chen, Y.Y. Li, and H.K. Chui, Wat Res, 30 (1996) 1353.
4. H.H.P. Fang, and G.-M. Zhou, J. Environ. Eng, 125 (1999) 57.
5. G.M. Zhou and H.H.P. Fang, Bioresource Tech, 61(1997) 47.
6. S S. Cheng, C.T. Ho and J.H. Wu, Wat. Sci. Tech, 36 (1997) 73.
7. R. Kleerebezem, J. Mortier, L.W. Hulshoff Pol, and G. Lettinga, Wat. Sci. Tech. 36(2-3) (1997) 237.
8. R. Kleerebezem, L.W. Hulshoff Pol, and G. Lettinga, Appl. Environ. Microbiol, 65 (1999a) 1152.
9. R. Kleerebezem, M. Ivalo, L.W. Hulshoff Pol, and G. Lettinga, Biotechnol. Prog, 15(3) (1999c) 347.
10. R. Kleerebezem, L.W. Hulshoff Pol, and G. Lettinga, Appl. Environ. Microbiol. 65 (1999b) 1161.
11. B.-R. Lin, Master Thesis, National Cheng-Kung University, 1995.
12. H. Macarie, A. Noyola and J.P. Guyot, Wat. Sci. Tech, 25(7)(1992) 223.
13. S.-J. Chu, Master Thesis, National Cheng-Kung University, 1997.
14. N.C. Tan, F.X. Prenafeta-Boldu, J.L.Opsteeg, G. Lettinga and J. A. Field, Appl Microbiol Biotechnol, 51 (1999) 865.
15. S.A. Reyes and G. Lettinga, Appl. Microbiol Biotechnol, 34 (1991) 544.
16. D.O. Mountfort and M.P. Bryant, Arch. Microbiol, 199 (1982) 249.
17. L. Schöcke and B. Schink, Microbiology 143 (1997) 2345.
18. B. Schink, Microbiol Mol Biol Rev, 61 (1997) 262.
19. H.H.P. Fang, H.K. Chui, and Y.Y. Li, Wat. Sci. Tech, 30 (12) (1994) 87.
20. F.A. MacLeod, S.R. Guiot and J.W. Costerton, Appl. Environ. Microbiol, 56 (1990) 1598.
21. R.I. Amann, B.J. Binder, R.J. Olson, S.W. Chisholm, R. Devereux and D.A. Stahl, Appl. Environ. Microbiol, 56 (1990) 1919.
22. R.I. Amann, W. Ludqig, and K.H. Schleifer, Microbial. Rev, 59 (1995)143.
23 S.M. Barns, R.E. Fundyga, M.W. Jeffries, and N.R. Pace, Microbiol, 91 (1994) 1609.
24. W.-T. Liu, T.L. Marsh, H. Chen and L.J. Forney. Appl. Environ. Microbiol. 63 (1997) 4516.
25. D.A. Stahl, Molecular Ecology, 4(1995) 535.
26. H.M. Harmsen, H.M.P. Kengen, A.D.L. Akkermans, A.J.M. Stams and W.M. De Vos, Appl. Environ. Microbiol, 62 (1996) 1656.
27. Y. Sekiguchi, Y. Kamagata, K. Syutsubo, A. Ohashi, H. Harada and K. Nakamura, Microbiology, 144 (1998) 2655.
28. F. von Wintzingerode, B. Selent, W. Hegemann and U.B. Gobel, Appl. Environ. Microbiol, 65 (1999) 283.
29. S. Rocheleau, C.W. Greer, J.R. Lawrence, C. Cantin, L. Larsme and S.R. Guiot, Appl. Environ. Microbiol, 65 (1999) 2222.

30. Y. Sekiguchi, Y. Kamagata, K. Nak amura, A. Ohashi and H. Harada. Appl. Environ. Microbiol, 65 (1999) 1280.
31. J. Dolfing and J.M. Tiedje, Appl Environ Microbiol, 54 (1988) 1871.
32. V.M. Warikoo. J. McInerney, J.A. Robinson, and J.M. Suflita. Appl. Environ. Microbiol. 62 (1996) 26.
33. H.H.P. Fang, H.K. Chui, and Y.Y. Li, Wat. Sci. Tech, 32 (8) (1995) 165.
34. G. Muyzer, E.C. de Waal, and A.G. Uitterlinden. Appl. Environ. Microbiol., 59 (1993) 695.
35. K. Smalla, U. Wachtendorf, H. He, W.-T. Liu and L.J. Forney, Analysis of BIOLOG GN carbon source utilization. Appl. Environ. Microbiol., 64(1998) 1220.
36. M. J. Ferris, G. Muyzer, and D.M. Ward, Appl. Environ. Microbiol, 62 (1996) 340.
37. A.T. Nielsen, W.-T. Liu, C. Filipe, L. Grady, Jr. R.S. Molin and D.A. Stahl, Appl. Environ. Microbiol, 65 (1999) 1251.
38. O.-C. Chan, unpublished results.
39. J.-H. Wu, unpublished results.
40. J.-H. Wu, W.T. Liu, and S.S. Cheng, in submission, 2000.
41. C.R. Woese, L. Achenbach, P. Rouviere and L. Mandelco, System. Appl. Microbiol, 14 (1991) 364.
42. J.T.C. Grotenhuis, C.M. Plugge, A.J.M. Stams, and A.J.B. Zehnder, Appl. Environ. Microbiol, 58 (1992) 1054.
43. J.E. Schmidt and B.K. Ahring, Biotech. Bioeng, 49 (1996) 229.
44. H.H.P. Fang, Y.Y. Li and H.K. Chui, J. Environ. Eng, 121 (1993) 153.
45. P. Hugenholtz, C. Pitulle, K.L. Hershberger and N.R. Pace , J. Bacteriol. 180 (1998) 4765.
46. M.A. Dojka, P. Hugenholtz, S. Haack, and N.R. Pace. Appl. Environ. Microbiol. 64(1998) 3869.
47. Y. Liu, D.L. Balkwill, H.C. Aldrich, G.R. Drake, D.R. Boone, Int. J. Syst. Bacteriol., 49 (1999) 545.
48. A.J.B. Zehnder, 1988. Biology of anaerobic microorganisms, Wiley, New Work.
49. O. Struck, O. Gross, B. Reichel, M. May, S. Hermann, N. Struckmann, B. Nonhoff, M. Lenke, A. Vilbig, J. Ludwig, A. Bode, K.H. Schleifer, and W. Ludwig, Department of Microbiology, Technische Universitat Munchen, Munich, Germany.

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§2 Health-Related Microorganisms

As the population grow and people inhabit in wider area, the distance between water resources and the sites of human activities is gradually narrowed. This is one of the reason why those pathogenic microorganisms, having been in waste streams without causing a human health-threat, suddenly have become a big issue.

The emergence of protozoa in water sources is a widespread problem in all over the world. Ohgaki *et al.* developed a method to estimate the health risks associated with the occurrence of *Cryptosporidium*. Based on the data taken from the Sagami River, he discusses that the risk of *Cryptosporidium* infection is more than 10-4, which increases slightly after the rainfall.

Modern methods developed for biotechnology is rapidly introduced into environmental microbiology. Polymerase-Chain-Reaction (PCR) can multiply a fragment of genes, thus has potential use for enumerating the unculturable or difficult-to-culture microorganisms. Katayama *et al.* applied a PCR method to count the number of coliphage Qβ. The PCR method was quite successful in counting the number of Qβ, but it gave a false number of viable count because it cannot differentiate those dead cells after disinfection by UV or chlorine from viable cells.

Algal bloom in water resources causes a variety of problems: e.g., bad odor and taste, troubles in water treatment, and algogenic toxins. Alam *et al.* presents the effect of UV radiation on the control of algae using *Microcystis aeruginosa* as a test species. Comparatively small dose of UV had negative impact on algal growth. UV radiation under the presence of organic matter showed a residual effect on growth inhibition of algae. This residual effect was correlated with the production of hydrogen peroxide and the presence of ferric iron.

Coliform and fecal coliform are widely used as indices of fecal pollution, and bacteriophages are proposed as surrogate indices for human viruses. Claydong *et al.* made an extensive survey on the presence of bacteriophages, coliform, and fecal coliform in wastewater from various sources. Three bacteriophage species, i.e., bacteriophage B, bacteriophage C, and F-specific bacteriophage were used as indicator organisms. Bacteriophage C was more abundant in all kinds of wastewaters than other bacteriophages, and had a better correlation with coliform count.

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Quantitative risk assessment of *Cryptosporidium* in a watershed

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In this study, an effort was made to assess the risk of infection of *Cryptosporidium* via drinking water. The field survey data from Sagami River Watershed were used for this purpose. The survey data indicates a continuous presence of *Cryptosporidium* in the water of Sagami River. The geometric mean concentration of oocysts in the 76 positive samples was 24 oocysts per 100L while 30 samples were negative. The maximum concentration was found to be as high as 11,000 oocysts per 100L. We used Monte Carlo simulations to calculate the annual risk of infection of *Cryptosporidium* via drinking water assuming the scenario that the sampling point of Katahara-Bashi or Miyayama was used as the water source of water purification plants. The risk for the two sampling spots were found to be higher than 10^{-4} infection per year. The incorporation of effect of rainfall increased the infection risk by a small fraction. However, during heavy rainfall the risk may increase considerably. Further study is required in this regard to evaluate the effect of rainfall more accurately.

1. INTRODUCTION

The outbreak of *Cryptosporidium* in Ogose, Saitama Prefecture, Japan, in 1996, has forced Japanese water supply utilities to take a whole new approach about risk management of pathogens. Occurrences of *Cryptosporidium* in drinking water were reported since 1983 in European countries and US. In 1998 in Australia, Sydney water supply authority was forced to suspend the water supply due to the occurrence of *Cryptosporidium* and *Giardia*. In the United States, on the average there are 3 to 5 outbreaks of infectious diseases per year [1]. In Japan, as shown in Table 1, although no cases of infection was reported after the Ogose outbreak, there are some instances that water supply were suspended when the *Cryptosporidium* was detected from 20L of finished water according to the guidelines of Ministry of Health and Welfare.

Of course, the risk of infectious disease transmission is the principle concern, but stoppage of water supply itself also poses serious risk on civil life. For example, lack of clean water for washing and cleaning, or anxiety of water shortage for fire extinguishing system can be major problems during the stoppage of water supply. Since the drinking water supply system are acting as a final barrier against disease transmission, its contamination by pathogens must be

Table 1.
Instances of Water Supply suspension due to Occurrence of *Cryptosporidium* or *Giardia*

Place	Population served	Protozoa*	Cases of infection	Date and Remarks
Saitama Prefecture Ogose-cho	approx. 14000	C	approx. 8800	June 1996, stop the purification plant and provided water from Saitama Prefecture
Fukui Prefecture Eiheiji-cho	362	G	0	7 July 1998 (Suspended)
Hyogo Prefecture Yumemae-cho	322	C	0	18 January 1999 (Suspended)
Yamagata Prefecture Asahi-mura	1553	C, G	0	15 July 1999 (Suspended)

* C for *Cryptosporidium* and G for *Giardia*

distinguished from the contamination of other foods, because user cannot help using the distributed tap water.

In this study, a comprehensive risk assessment was undertaken based on the field data collected from Sagami River watershed [2]. Risk of infection of *Cryptosporidium* via drinking water was calculated following the ILSI/RSI quantitative risk assessment framework [3]. In addition to the source water parameters, the effect of the rainfall was also incorporated in the annual risk calculation. There have been some report that concentration of *Cryptosporidium* increases during rainfall [4]. Since rainfall is relatively frequent in Japan, as is common in the Asian monsoon region, we also assessed its effect on the annual risk.

2. MATERIAL AND METHODS

2.1 Field Survey

The procedures for collection of data and enumeration of *Cryptosporidium* have been described in detail in another paper [2]. Important parts of that paper are repeated here for convenience. Detection of *Cryptosporidium* oocysts was done following the guidelines of Ministry of Health and Welfare, Japan [5].

2.1.1 Sample collection

The Sagami River and its two tributaries (Koayu and Nakatsu Rivers) in Kanagawa prefecture in Kanto area of Japan were selected for the study (Figure 1). The Sagami River flows from the Lake Yamanaka near Mt. Fuji to Sagami Bay. It has two reservoirs in the upstream, and it passes through urban areas such as Atsugi and Sagamihara in the middle. Some tributaries such as Nakatsu and Koayu from the Tanzawa mountain system join in the middle. The Sagami River is one of the main water sources in Kanagawa prefecture, including Yokohama City. From April 1997 to June 1998, a total of 106 samples were collected at 11 sites along the river which include 2 water intake points, Miyayama, in the downstream of Sagami River, and Hambara, in the upstream of Nakatsu River. Samples from these sites were collected one or two times every two months.

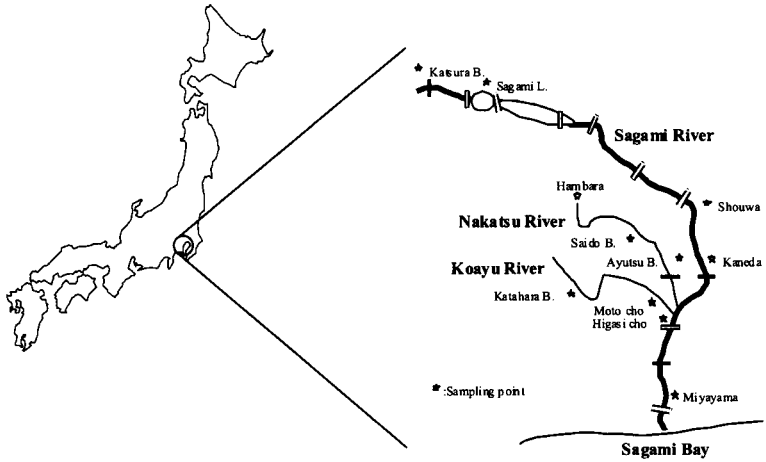

Figure 1. Location of sampling sites in the Sagami River and its tributaries

2.1.2 Concentration and purification

Samples were brought to the laboratory and were filtered in the same day. Samples of 100 liters were filtered through cellulose acetate membrane disk filters (142mm diameter, 1.2 μ m pore size, Millipore). Then filters were completely dissolved in acetone in centrifuge tubes and centrifuged at 1050g for 10minutes. The pellet was purified by percoll-sucrose floatation.

2.1.3 Staining procedure

The purified samples were filtered using 25mm diameter membrane disk filters. An indirect fluorescent antibody (IFA) stain was applied to the purified samples on the filter using a *Cryptosporidium* antibody kit (Hydroflour combo kit, SDI, USA).

2.1.4 Identification and counting

Filters were observed at 200 and 1000 times magnification by epi-fluorescent and differential interference contrast (DIC) microscope (BX-60, Olympus, Japan). The identification was carried out following the procedure of U.S.EPA (1998) [6]. *Cryptosporidium* oocysts were detected by fluorescent shape and size and confirmed by the presence of sporozoite(s) or surface folds.

2.2 Risk Analysis

2.2.1 Effect of rainfall

It is generally believed that rainfall volume has significant effect on the *Cryptosporidium* concentration of the raw water that is used in water purification plants. To find out the relationship between rainfall and *Cryptosporidium* concentration of the raw water, the data

from the Miyayama sampling spot in the Sagami River was used. *Cryptosporidium* concentration was plotted against the rainfall volume.

2.2.2 Risk assessment

Risk of infection of *Cryptosporidium* via drinking water was calculated following the ILSI/RSI quantitative risk assessment framework [3]. To incorporate the effect of rainfall on the annual risk of infection, we improved the procedure to be detailed in Figure 2. The data (only rainless days) from the various sampling points were first fitted to log-normal distribution to obtain the concentration of *Cryptosporidium*. The relationship between rainfall and *Cryptosporidium* concentration (described in section 2.2.1) was then used to modify the concentration of *Cryptosporidium*. The observed rainfall data of 1998 was used in this study as a model for predicting the rainfall. The effect of viability of the oocysts was then incorporated using data from Teunis *et al.* (1997) [7]. A beta distribution was assumed with values of α and β of 1.65 and 2.46 respectively. Removal by coagulation and filtration was represented by a two choice binomial model: either good removal (median = 99.6%) or a degraded performance (median = 70.6%, 1.5% of occurrence). Effect of disinfection on the viability of the oocysts was not considered, because chlorination is known to produce negligible effect. Consumption of unboiled drinking water was also considered to have variability and to follow a log normal distribution with a median value of 0.153 l/day [3]. Dose-response relationship was based on the data obtained by DuPont, *et al.* (1995) [8]. Monte Carlo simulation was then used to calculate the infection risk of a year, and 1000 years were computed and analyzed statistically.

3. RESULTS AND DISCUSSIONS

3.1 Field Survey

The field survey data obtained during 1996-1997 are described in detail in another paper [2]. The results are summarized in Table 2. Of the total 106 samples, *Cryptosporidium* oocysts were found in 76 samples. The geometric mean concentration of the positive samples were 24 oocysts/100L (range = 1 to11,000 oocysts/100L).

In Sagami River, *Cryptosporidium* oocysts were found in 41 of the 56 samples from 6 sites. The geometric mean concentration was found to be 9 (range=1 to 710) oocysts per 100L.

In Nakatsu River, *Cryptosporidium* oocysts were detected in 14 of the 29 samples from 3 sites. The geometric mean was 9 (range 1 to 500) oocysts per 100L.

In Koayu River, 21 samples from 2 sites were examined and *Cryptosporidium* oocysts were detected in all the samples. The geometric mean of the samples were 320 (range 6 to 11000) oocysts per 100L.

The one year long survey demonstrated the continuous pollution of *Cryptosporidium* oocysts in Sagami River and its tributaries, but no seasonal effect was observed.

The highest concentrations of *Cryptosporidium* oocysts were observed in the sample collected from Katahara Bridge, Koayu River, where waste water with insufficient treatment were discharged from a swine farm. The concentration of *Cryptosporidium* oocysts varied from 210 to 11000 oocysts per 100L with a geometric mean of 1500. In order to confirm the source of the protozoan pollution, the concentrations of oocysts in the in the swine wastewater from the farm was investigated. High concentration of *Cryptosporidium* oocysts (range=1000 to 1000 oocysts/L, geometric mean=2,200 oocysts/L) were found.

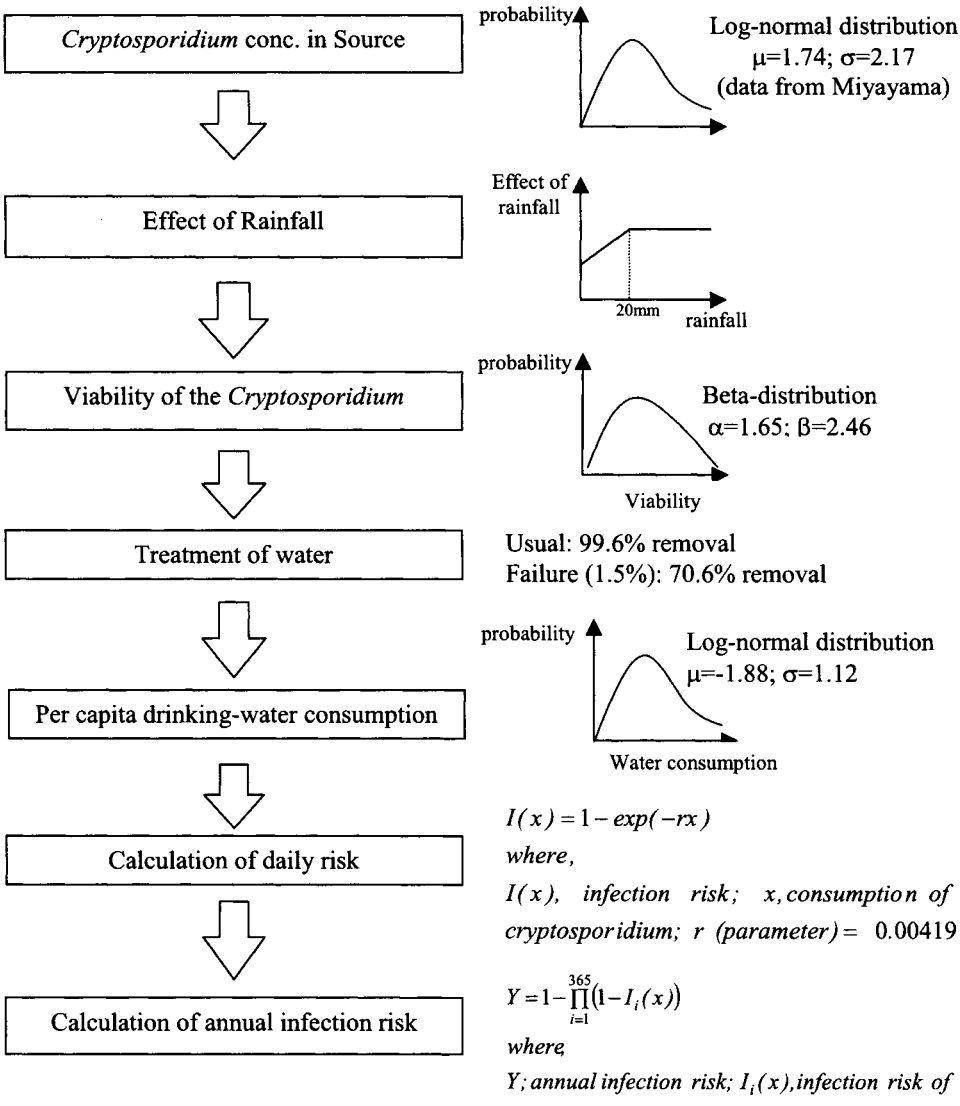

Figure 2. Procedure for assessment of risk of *Cryptosporidium*

Table 2.
Occurrence of *Cryptosporidium* oocysts in Sagami Watershed. [2]

	Cryptosporidium oocysts	
	GM*(range) oocysts/100L	Positive ratio**
Sagami River		
Katsura B	3 (3-3)	1/1
Sagami L	5 (1-22)	5/8
Miyayama	10 (1-180)	23/26
Shouwa B.	3 (1-9)	4/7
Kaneda	7 (7-7)	1/7
Higashi Cho	23 (5-710)	7/7
Total of Sagami River	9 (1-710)	41/56
Nakatsu River		
Hambara	1 (1-1)	1/12
Saido B	5 (1-23)	8/12
Ayutsu B	31 (3-500)	5/5
Total of Nakatsu River	9 (1-500)	14/29
Koayu River		
Katahara B	1,500 (210-11000)	12/12
Moto Cho	39 (6-100)	9/9
Total of Koayu R	320 (6-11000)	21/21
Total	24 (1-11000)	76/106

*GM = geometric mean concentration
**Positive ratio= Number of positive sample/total number of sample

The sampling sites of Hambara (Nakatsu River) and Miyayama (Sagami River) are located near the water intake point of source water for Kanagawa Prefecture. In Hambara, in upstream of Nakatsu River, *Cryptosporidium* oocysts were detected from only one of the 12 samples and the concentration was only 1 oocyst per 100L. But *Cryptosporidium* oocysts were found in 23 of the 26 samples in the Miyayama site. The geometric mean concentration was 10 oocysts per 100L (range = 1 to 180 oocysts per 100L).

3.2 Risk Of Infection By *Cryptosporidium* Oocysts

3.2.1 Effect of rainfall on *Cryptosporidium* concentration

Cryptosporidium concentration at any day in the raw water was found to be correlated (r^2=0.6639) with the rainfall at that particular day of sampling (Figure 3). The regression line was drawn only using the data of the *Cryptosporidium* concentration against the rainfall on that particular day. Although the figure shows that successive days of rainfall may increase the *Cryptosporidium* concentration, those data were not used in the regression analysis. However, practically the *Cryptosporidium* concentration can not increase with rainfall beyond a certain level; hence we assumed in the risk calculation that above a rainfall intensity of 20 mm/day (the maximum rainfall at which the *Cryptosporidium* concentration was measured), *Cryptosporidium* concentration remains the same.

3.2.2 Risk of infection incorporating the effect of rainfall

The annual risk of infection of *Cryptosporidium* through drinking water in Miyayama and Katahara-Bashi are shown in Figure 4, Figure 5 and in Table 3.

We can see from the Table 3 that the median annual risks in Miyayama and Katahara-Bashi are $10^{-3.68}$ and $10^{-1.12}$ respectively. Both of these values are higher than the acceptable annual risk of 10^{-4}, which was proposed by Haas *et al.* (1996) [9]. One way to reduce the risk is suspending the water supply. As a further study, we calculated that if water supply is suspended whenever *Cryptosporidium* level reach a concentration of 1 oocysts per 80L, the annual infection risk for Miyayama can be reduced to the acceptable level. However, further study is required in this direction about the risk involved in suspending the water supply.

Quantitative microbial risk assessment is an invaluable tool for assessing consequences of exposure to infectious microorganisms. Risk assessment helps in developing necessary precautions to mitigate or control such consequences. However, a lot of uncertainties are involved in microbial risk assessment. The dynamic nature of infectious diseases, substantial biological variability in both hosts and pathogens and the nature of microbiological analysis make it imperative to use stochastic methods for the characterization of risks. This requires a vast amount of information of a highly detailed level. However, detailed data are hardly available. For example, for assessing the daily ingested dose of viable oocysts, data regarding the concentration of oocysts in the raw water, viability of the oocysts, reduction by treatment and disinfection and consumption of water are required. Each of these data sets should include detailed information; for example, raw water concentration of oocysts should include source of the oocysts i.e., those, which are infectious to humans, and those, which are not. Reduction by physical treatment also involves lot of uncertainties. The low and variable recovery of current detection methods in relation to low concentrations of oocysts in water precludes such observations. Hence, critical information on the relation between oocyst removal and surrogate parameters should be gathered under variety of circumstances. However, at present there is little information regarding the effect of water quality on the

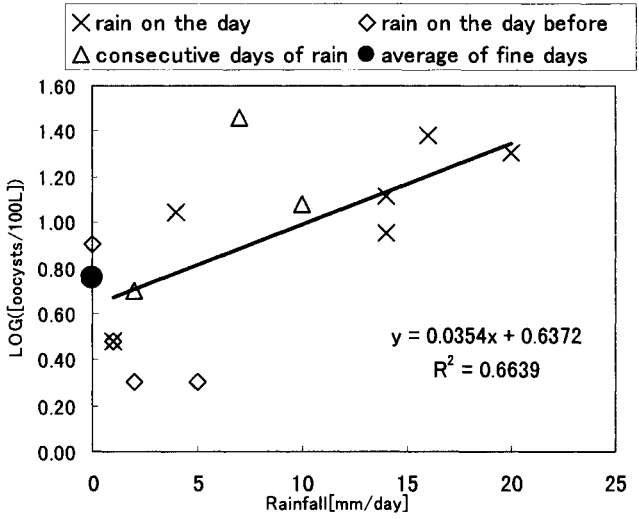

Figure 3. Effect of rainfall on *Cryptosporidium* concentration

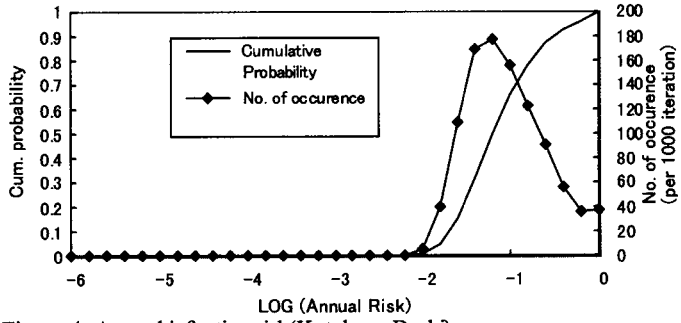

Figure 4. Annual infection risk(Katahara-Bashi)

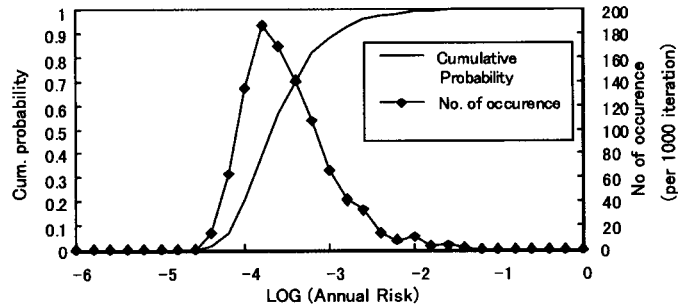

Figure 5. Annual infection risk(Miyayama)

Table 3.
Annual risk of *Cryptosporidium* infection (Values are in 10-base logarithm)

	Miyayama	Katahara-Bashi
Geometric Mean	-3.60	-1.14
Median	-3.68	-1.12
Maximum	-1.44	0.00
Minimum	-4.53	-2.10
95% confidence value	-2.56	-0.281

physical removal of *Cryptosporidium*. Reliable data on the consumption of raw tap water is also unavailable. Another important field where there is a considerable lack of information is the host pathogen interaction. The interaction between human hosts and pathogenic microorganisms is a highly complex and variable process and detailed information is needed to accurately model this stage. However, there are only limited information available in this regard and the information do not include the susceptibility of immunologicaly different groups.

4. CONCLUSIONS

Our study indicates a continuous presence of *Cryptosporidium* in the water of Sagami River. The geometric mean concentration of oocysts in the 76 positive samples was 24 oocysts per 100L. The maximum concentration was found to be as high as 11,000 oocysts per 100L. Swine wastewater was found to be one of the major contributors of the *Cryptosporidium* pollution in the Sagami River. We used Monte Carlo simulations to calculate the annual risk of infection of *Cryptosporidium* via drinking water assuming the scenario that the sampling points of Katahara-Bashi and Miyayama being used as the water source of water purification plants. We further assumed that the raw water is subjected to coagulation, flocculation and chlorination. In such cases we found that the annual risk of infection of *Cryptosporidium* via drinking water for the two sampling spots would be higher than 10^{-4}. The incorporation of effect of rainfall increased the infection risk by a small fraction. However, during heavy rainfall the increase in risk may be considerable. Further study is required in this regard to evaluate the effect of rainfall more accurately.

REFERENCES

1. Haas C.N., Rose, J.B., Gerba C.P. (1999) Quantitative Microbial Risk Assessment, John Willey & Sons, Inc, New York.
2. Hashimoto A. and Hirata T. (1999) Proceedings of Asian Waterqual '99: 7th IAWQ regional conference, Vol.2, pp. 956-961.
3. Teunis P.F.M. and Havelaar A.H. (1999). RIVM Report no. 284550006.
4. Atherholt T.B., Lechevallier M.W., Norton W.D., Rosen J.S. (1998) Journal of American Water Works Association, 90(9), pp. 66-80.
5. Ministry of Health and Welfare, Japan. (1996, 1998) Tentative Guidelines for Protection of Water Supply from Cryptosporidium Pollution.
6. U.S. EPA (1998), Method 1622: U.S.EPA
7. Teunis P.F.M., Medema G.J., L. Kruidenier and A. H. Havelaar (1997) Water Research, 31(6), pp. 1333-1346.
8. Dupont H.L., Tager I.B., Sterling C.R., Okhhuysen P.C., Rose, J.B., Jakubowski W. (1995) The New England Journal of Medicine, 16(4), pp. 855-859.
9. Haas C.N., Crockett C.S., Rose, J.B., Gerba C.P. and Fazil A.M. (1996) Journal of American water works association, 88, 131-136.

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PCR determination of inactivated RNA coliphage Qβ

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Polymerase chain reaction (PCR) may provide positive results regardless of infectivity of viruses, and it is of concern in the monitoring of pathogens after disinfection in water and wastewater treatment systems. F-specific RNA coliphage Qβ (ssRNA=4217bp) was used as a model virus, and disinfected by chlorination and UV irradiation. In order to reduce the positive results from noninfective viruses with damaged genomic RNA, reverse transcription region was elongated to be applied as well as normal RT-PCR to the disinfected samples. The normal RT-PCR method had an amplicon of 193bp, while the long RT-short PCR method had the RT region of 1909bp and the PCR amplicon of 244bp. MPN method was applied to quantify the results of these two RT-PCR methods. The MPN values of the UV irradiated Qβ were almost same as that of the intact Qβ. This means that the RT-PCR detects quantitatively almost all noninfectious RNA coliphages inactivated by UV radiation. High dose (0.9 mgCl/L) chlorination reduced the MPN value of normal RT-PCR by 4log in 60 min while decreasing the plaque counts by 4log in 1min. On the other hand, the MPN values of both PCR methods were almost constant during the low dose (0.4 mgCl/L) chlorination for 60 min while the plaque counts showed 7log decrease in 30min. Since the free chlorine almost disappeared and converted to combined chlorine in 1 min in low dose chlorination, combined chlorine did almost no damage to the viral RNA.

1. INTRODUCTION

To protect public health, monitoring of pathogenic viruses is necessary in water supply and wastewater systems. The polymerase chain reaction (PCR) is one of the feasible monitoring methods, because it requires less than 6 hours and no cultivation of pathogenic viruses but detect viruses sensitively. In recent years, PCR has been used to detect enteric viruses in environmental samples [1-7]. One of the difficulties in using PCR for virus monitoring is that PCR may not

reflect infectivity of viruses [8] and may fail to evaluate the health risk. The positive RT-PCR results of viruses from environmental sample could reflect the existence of intact viruses since RNA was unstable in wastewater [3] or seawater [9]. Possibility of detecting only intact viruses was increased by the use of antibody [10, 11] or applying cell culture prior to RT-PCR [12]. However, disinfection in water and wastewater treatment may still cause false-positive results in RT-PCR detection of viruses. Reduction of concentration of viruses by monochloramine [13], free chlorine and UV irradiation [14] was estimated from the detection of decimal dilution series, which was not quantitative enough. In this study, F-specific RNA coliphage was used as a model virus of enteric viruses and was quantified after chlorination and UV irradiation by RT-PCR and plaque assay.

2. MATERIALS AND METHODS

Preparation of Qβ: The F-specific RNA coliphage Qβ was prepared from the lysate broth of single-plaque-isolated stock. The plaque assay method used was double-agar-layer method using *E.coli* K12, F^+, A/λ as the host strain [15].

UV radiation: The Qβ stock was diluted one to 400th by sterilized pure water to reduce the absorbance of 260nm to 0.15/cm, and 35ml was applied in a petri dish of diameter 5.6 cm. The petri dishes were placed at 10 cm from a low-pressure mercury UV lamp (15W) and continuously stirred by a magnetic bar during UV irradiation. The UV intensity induced to the sample was measured using the Qβ survival ratio method [16].

Chlorination: Qβ containing broth media was injected into NaClO solution in a sterilized glass bottle with a cap or disposable tube, in which the broth media was diluted 10,000 times, and chlorinated for 60min. The initial free chlorine concentration ranged from 0.3 to 1.0 mgCl/L. Diethyl-p-Phenylene Diamine (DPD) or Ortho-Tolidine (OT) method was applied to the determination of free and combined chlorine concentration. After neutralization with $Na_2S_2O_3$, chlorinated Qβ was subjected to plaque assay and two kinds of RT-PCR methods.

RNA extraction: The direct heating method was used for extracting RNA of Qβ coliphage in a sample. One hundred microliters of phage was added into a 0.5ml Eppendorf tube and heated 10 minutes at 90°C then cooled quickly down to 4°C.

Primers: The primers for detecting Qβ were the sequences for amplifying the 3' terminal region (setI ,setII) or 5' terminal region (setIII ,setIV) on the replicase gene of Qβ RNA genome and specific to F-specific RNA coliphage group III [17]. The sequences of primers used were;

setI-: 5'TTTATTCACAATTAGGCGCCAT3', setI+ : 5'CCATCGATCAGCTTATCTGTA3',

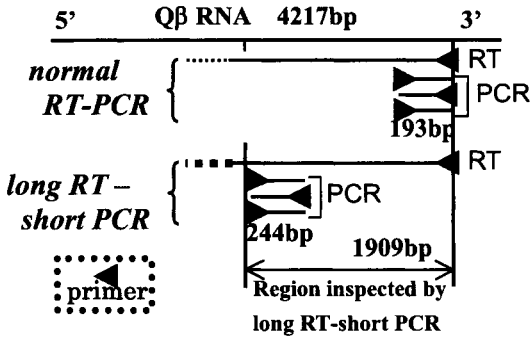

Figure.1 Schematic of long RT-short PCR

setII- : 5'CTCGTGTAGAGACGCAACCTT3', setII+ : 5'GTCTACCGGCAAATTCGATAT3', setIII- : 5'AGAGTTAAATGGCGACTGACCAT3', setIII+ : 5'GTGCCATACCGTTTGACTTCACT3', setIV- : 5'GTGCGAGGTTACCTTCAACCT3', setIV+ : 5'CAAGCCGTGATAGTCGTTCCT3'.

Reverse Transcription: Three microliters of direct heating-extracted RNA sample were added into 17μl of RT mixture. The RT mixture included 10mM Tris-HCl (pH 8.3), 50mM KCl, 5.25mM $MgCl_2$, 1.0mM dNTPs, 1.0μM setI- primer, and 0.375U/μl AMV Reverse Transcriptase XL in total 20μl. The mixture was overlaid with 40μl of mineral oil and incubated 30 minutes at 42°C, heated at 99°C for 3 minutes and quickly cooled down to 4°C.

Polymerase chain reaction(normal): To each tube after RT reaction, PCR mixture was directly added to obtain 100μl final volume. The PCR condition was 10mM Tris-HCl(pH 8.3), 50mM KCl, 1.5mM $MgCl_2$, 1.0mM dNTPs, 0.2μM setI- primer, 0.2μM setI+ primer, and 0.025U/μl Taq polymerase. The reaction tubes were incubated 3 min at 95°C, then 25 or 30 cycles of 95°C for 30sec, 50°C for 30sec, 72°C for 30sec, then finally 7min at 72°C and cooled down quickly to 4°C. The length of amplicon was 193bp, while the total length of Qβ genome is 4217bp.

Nested PCR (normal): Fifty microliters of nested PCR mixture were prepared in each new tube. The mixture included 9.6mM Tris-HCl (pH 8.3), 48mM KCl, 1.4mM $MgCl_2$, 0.15mM dNTPs, 0.2μM setII- primer, 0.2μM setII+ primer, and 0.02U/μl Taq polymerase. Five microliters of sample after PCR were added into each nested PCR mixture to obtain 55μl final volume. The amplicon was 102bp. The thermal protocol was the same as first PCR of 25 cycles.

Long RT-short PCR: The outline of long RT - short PCR method is shown in Figure 1. The protocol of long RT-short PCR was the same as normal RT-PCR until RT reaction with primer setI-. In the first and nested PCR, primer setIII and setIV were used respectively, in order to confirm that RNA were reverse-transcripted through the replicase gene of Qβ RNA. The length of the RT region,

the PCR amplicon and the nested PCR amplicon was 1909bp, 244bp and 141bp, respectively.

Analysis of PCR product: Analysis of PCR products was conducted by gel electrophoresis [4]. The band of the designed length on the agarose gel was defined as positive detection. MPN method, decimal series of three or more dilutions with five portions [18], was applied to quantify the PCR results.

Determination of RT region in long RT-short PCR method: In order to prove that RT reaction was originated from the annealing point of primer setI-, the yields of cDNA with 5 non-specific primers in RT reaction was determined by nested PCR using the primers setIII and setIV. The primer setIII- was also applied in RT reaction for the PCR efficiency using the primers setIII and setIV.

3. RESULT AND DISCUSSION

3.1 Sensitivity test

The MPN values and their ratios to plaque counts of Qβ stock are shown in Table 1. The MPN/PFU ratios are defined as the MPN value divided by the plaque counts per milliliter. Since the ratios obtained from normal RT-PCR had geometric mean value of 0.32 and the standard deviation of its logarithm was 0.07, ratio can be written as $10^{-0.48 \pm 0.07}$. This result showed the MPN value from normal RT-PCR had enough reproducibility and high sensitivity. On the other

Table 1
Ratio of MPN and PFU

	PFU/ml	Dilution factor				MPN/ml	MPN/PFU
		10^{-5}	10^{-6}	10^{-7}	10^{-8}		
normal RT-PCR	1.2×10^{10}	5/5	5/5	3/5	-	3.1×10^{9}	**0.26**
	1.2×10^{10}	5/5	5/5	4/5	0/5	4.3×10^{9}	**0.36**
	1.2×10^{10}	5/5	5/5	5/5	1/5	1.2×10^{10}	**0.33**
	1.6×10^{10}	5/5	5/5	4/5	1/5	5.8×10^{9}	**0.36**
						Geometric Mean	**0.32**
long RT-short PCR	1.6×10^{10}	5/5	2/5	0/5	0/5	1.6×10^{8}	**0.01**
	1.2×10^{10}	5/5	3/5	0/5	-	2.6×10^{8}	**0.022**
	3.6×10^{10}	5/5	5/5	4/5	1/5	5.8×10^{9}	**0.16**
						Geometric Mean	**0.033**

hand, Since the ratios obtained from long RT-short PCR had geometric mean value of 0.033 and the standard deviation of its logarithm was 0.62, the ratio can be written as $10^{-1.48\pm0.62}$. The MPN value from long RT-short PCR

3.2 Determination of RT region in long RT-short PCR method

The detection sensitivity of RT-PCR using setIII- as RT primer was 0.2 PFU/tube, which is almost the same as normal RT-PCR. On the other hand, in the 5 cases using non-specific primer in place of RT primer, the lowest Qβ concentrations that had positive result were more than 10^5PFU/tube. The lowest concentration with positive result of long RT-short PCR was 10^1PFU/tube. These results suggests that almost all the cDNA synthesized in the RT reaction were not products of non-specific annealing of primer setI- but from specific annealing. In the long RT-short PCR method, therefore, the template RNA should be the position between the place specific to primer setI- and the PCR target region. Since the condition of RT reaction was same in the both RT-PCR and the sensitivity of setIII and setIV PCR was almost same as normal RT-PCR, the long RT had lower recovery yields of cDNA than normal RT-PCR.

3.3 Detection of Qβ disinfected by UV

The inactivation curve of Qβ by UV radiation is shown in Figure 2. After 15 min irradiation, the survival ratio became $10^{-7.5}$. The intensity of UV radiation calculated from the inactivation rate of Qβ was 110 μW/cm^2. The amount of UV dose in 30 min was 200,000 μWs/cm^2, where the survival ratio of Qβ was

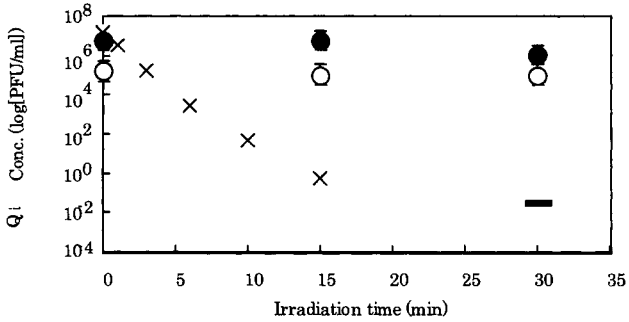

Figure 2. Inactivation of Qβ by UV irradiation and RT-PCR determination of Qβ genomic RNA

× plaque assay [PFU/ml]
● normal RT-PCR [MPN/ml]
○ long RT-short PCR [MPN/ml]
▬ not detected

calculated to be 10^{-15}. No difference was observed in MPN values of normal RT-PCR by 200,000 μWs/cm^2 of UV irradiation. From the theoretical consideration, the length of template RNA of normal RT-PCR (190bp) and that of long RT-short PCR(1909bp) were 4.5% and 45% of Qβ genomic RNA (4217bp), respectively. Assuming that the RNA template with a pyrimidine dimer produced by UV irradiation could not be reverse-transcripted, the long RT-short PCR would show half amount of decrease of plaque counts did after UV irradiation. Hence, the reverse-transcription was not terminated by the dimers produced by UV irradiation.

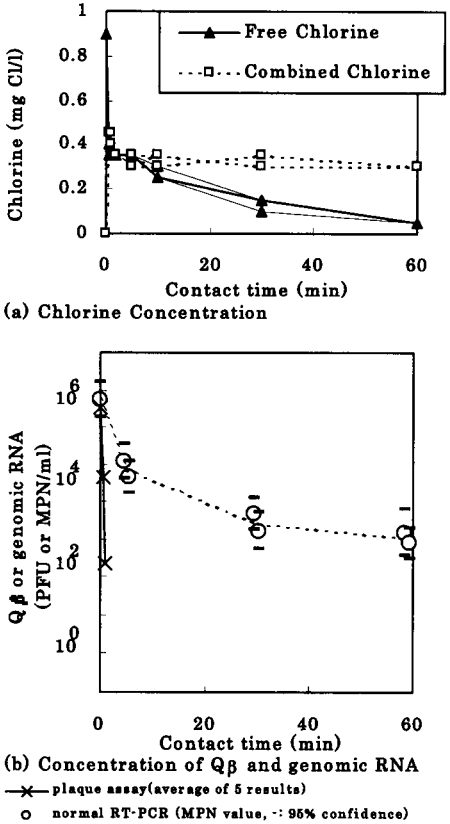

Figure 3. The concentration profile of chlorine and Qβ in 0.9 mgCl/L dose

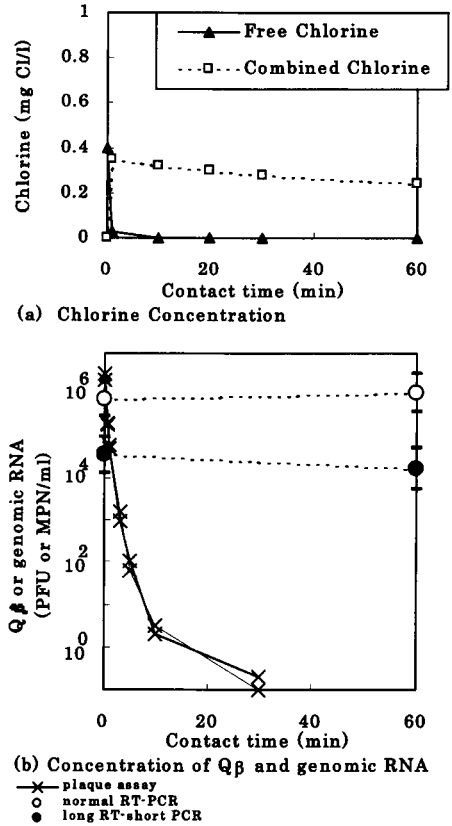

Figure 4. The concentration profile of chlorine and Qβ in 0.4 mgCl/L dose

3.4 Chlorination of Qβ

The concentration profiles of chlorine and Qβ in high (0.9 mgCl/L) dose case and those in low (0.4 mgCl/L) dose case are shown in Figure3 and Figure 4, respectively. The free chlorine in high dose case remained for 60 min, while in low dose case the free chlorine almost disappeared and was converted to combined chlorine in 1 min. The MPN values by the normal RT-PCR method decreased by 4log in 60 min in high dose chlorination, while the plaque counts showed 4log decrease in 1 min. These results were in consistent with previous report on poliovirus disinfected by free chlorine [14]. Long RT-short PCR was not tested because its sensitivity was not enough for detection after high dose chlorination. The MPN values of the normal RT-PCR method and of the long RT - short PCR method showed no decrease in low dose chlorination, while the plaque assay results showed 7log decrease in 30 min. Free chlorine remained in the solution (Figure 3) and damaged the target site of normal RT-PCR method.

4. CONCLUSION

MPN enumeration was applied to the result of RT-PCR detection, which proved to be successfully available for quantification of RNA coliphage Qβ. The long RT-short PCR had long RNA template, though it provide lower MPN values by 10 times than normal RT-PCR.

Both normal RT-PCR and long RT-short PCR detected quantitatively the all noninfectious Qβ damaged by UV radiation as positive.

Free chlorine destroyed Qβ genomic RNA to reduce the MPN values of normal RT-PCR. Both normal RT-PCR and long RT-short PCR detected quantitatively the all noninfectious Qβ disinfected by combined chlorine as positive.

REFERENCE

1 Jothikumar N., K. Aparna, S. Kamatchiammal, R. Paulmurugan, S. Saravanadevi and P. Khanna. 1993. Appl. Environ. Microbial. 59:2558-2562

2 Kopecka H., S.Dubrou, J.Prevot, J. Marechal, and Lopez-Pila. 1993. Appl. Environ. Microbial. 59:1213-1219

3 Limsawat S., and Ohgaki S. 1997. Appl. Environ. Microbial. 63:2932-2933

4 Limsawat S., Kamiko N., and Ohgaki S. 1995. Wat. Sci. Tech. 31:383-390.

5 Puig M., J.Jofre, F. Lucena, A. Allard, G. Wadell, and R. Girones. 1994 Appl. Environ. Microbial. 60:2963-2970

6 Schwab K. J., R. De Leon, and M. D. Sobsey. 1993. Wat. Sci. Tech. 27:211-218

7 Tsai Y., M. D. Sobsey, L. R.Sangermano, and C. J. Palmer. 1993. Appl. Environ.

Microbial. 59:3488-3491
8 Alexander L. M. and Morris R. 1991. Wat. Sci. Tech., 24: 291-294.
9 Tsai Y, B. Tran and C. J. Palmer. 1995. Appl. Environ. Microbial. 61:363-366
10 Schwab K. J., R. De Leon, and M. D. Sobsey. 1996. Appl. Environ. Microbial. 62:2086-2094
11 Jothikumar N., D. O. Cliver, and T. W. Mariam. 1998. Appl. Environ. Microbial. 64:504-508
12 Reynolds K. A., C. P. Gerba and I. L. Pepper. 1996. Appl. Environ. Microbial. 62:1424-1427
13 Shin G. and M. D. Sobsey. 1998. Wat. Sci. Tech., 38: 151-154.
14 Ma J. T. M. Straub, I. L. Pepper and C. P. Gerba. 1994. 4203-4206
15 Ketratanakul A. and Ohgaki S. 1989. Wat. Sci. Tech.,. 21: 73-78.
16 Kamiko N. and Ohgaki S. 1989. Wat. Sci. Tech., 21: 227-231
17 Danteravanich S. 1992. Doctoral dissertation, The Univ. of Tokyo, Tokyo, Japan.
18 Hurley M. A. and M. E. Roscoe 1983. Journal of Applied Bacteriology, 55: 59-164.

Evaluation of UV-radiation and its residual effect for algal growth control

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In this study, the scope of using UV-radiation to control algal growth was assessed using *Microcystis aeruginosa* as test species. A UV-dose of 75-mWs/cm^2 was found to be lethal to *M. aeruginosa*. A smaller dose of 37-mWs/cm^2 prevented growth for about 7-days. Our study clearly showed that UV-radiation might produce residual effect that was harmful to microorganisms (*M. aeruginosa* and *E. Coli.*). It was also shown that the residual effect might persist for long duration. The extent of residual effect increased with increasing UV-dose. However, residual effect of UV-radiation is highly dependent on the constituents of the irradiated water. Presence of organic matter which can act as photosensitizer is essential for the residual effect of UV-radiation. We found that, UV-radiation can produce μM level of H_2O_2 in the irradiated water and the H_2O_2 production increases with increasing UV-dose. The H_2O_2, that was produced due UV-irradiation showed a first order decay (half-life of about 7-days) while stored in the dark at 4^{0}C. But when H_2O_2 was externally added to the same synthetic water, there was almost no change in H_2O_2 concentration during 7-days of storage under the same conditions. These results suggest the presence of reactive species which react with H_2O_2 in the irradiated water. It was also found that the extent of residual effect as well as the H_2O_2 production increases in the presence of metal ions (Fe^{3+}). Hence we concluded that both H_2O_2 and metal ions play significant role in producing the residual effect of UV-radiation.

1. INTRODUCTION

Ultraviolet irradiation is a potent biocide; its effectiveness against various groups of microorganisms is well documented [1-5]. However, there is little information about the inactivation of algae by UV-radiation. Inactivation of algae by copper sulfate (in the lakes and reservoirs) is detrimental to the ecology of the lake [6]. In water treatment facilities algae increases the THMFP (trihalomethane formation potential) if ozone or chlorine is used as pre-disinfectant [7]. In this study we tried to explore the scope of using UV-radiation for management of algal-growth related problems in water resources facilities. Another important phenomenon, namely the residual effect of UV-radiation, was also studied. Previously, it was believed that UV-radiation does not produce any harmful disinfection by-product. But recently, some researchers [8,9] have shown that UV-radiation may produce significant residual effect. Lund, *et al.*, 1994 [10], have suggested that UV-radiation may produce H_2O_2 which might be responsible for the residual effect. But there is no published data about the

the concentration and fate of this UV-induced H_2O_2. So, we measured the H_2O_2 production due to UV-radiation and attempted to determine its role in producing the residual effect.

2. MATERIALS AND METHODS

In this study, the scope of using UV-radiation to control algal growth was assessed using *Microcystis aeruginosa* as test species. *M. aeruginosa* was selected for the experiments because of its frequent association with seasonal algal bloom. The residual effect of UV-radiation, was studied using *M. aeruginosa* and *E. coli* as test species. Synthetic water containing various combinations of organic matter, metals and inorganic nutrients was irradiated under low-pressure mercury lamps. After irradiation, *M. aeruginosa* and *E. Coli* were inoculated in the irradiated water and their growth was monitored.

2.1 Cultivation of algae

Axenic culture of blue green algae *Microcystis aeruginosa* was obtained from National Institute for Environmental Studies (NIES), Japan and was then grown in standard culture media (CT-media [11]). The pH was adjusted to 9.0 by buffering with bicine (N,N-2Bis(2-hydroxymethyl) glycine $C_6H_{13}NO_4$) instead of Tris (hydroxymethyl) aminomethane. Cultures were maintained at 25°C in an incubation chamber with controlled humidity and lighting. Fluorescent lamps (FL20SW-B, GE/Hitachi) were used as the light source with an automated 16h/8h-light/dark cycle. The light intensity during the light phase was 1500-lux.

2.2 Effect of direct UV-radiation

Samples were prepared by inoculating exponentially growing cells into autoclaved media to give an initial concentration of about 10^5 cells/ml. To assess the effect of direct UV-radiation (254 nm), 40-ml samples were irradiated in petridishes (depth of the sample = 0.67 cm) for 30 seconds to 2 minutes. The intensity of UV-radiation at the surface of the sample was measured by a UV-radiometer (Topcon Corp., Japan) and the intensity varied from 1 to 1.5-mW/cm^2. Irradiation was done either by a single low-pressure mercury lamp (National Co. Ltd., GL-10, 10W), or by two low-pressure mercury lamps (Toshiba corp., GL-20, 20W each). The UV-dose was calculated using Beer-Lambert law. After irradiation, the samples were incubated in an incubation chamber (1500-lux fluorescent light, 25°C temperature, 16h/8h-light/dark cycle) in 60-ml Pyrex glass tubes. The growth was measured by counting cells in haemocytometers by a phase contrast microscope. Only those cells that showed no visible damage were counted, i.e., broken and faded cells were considered dead. All growth experiments were carried out in triplicate.

2.3 Residual effect on *M. aeruginosa*

To study the residual effect of UV-radiation on algae, the standard culture media for *M. aeruginosa* (JWWA, 1993) was irradiated under two low-pressure mercury lamps (Toshiba corp., GL-20, 20W each) for 1 to 3 minutes to produce UV-doses of 100, 200, 400 and 800 mWs/cm^2. Intensity of radiation at the surface was varied between 1.5-mW/cm^2 to 6-mW/cm^2 by changing the distance between the surface of the liquid and the lamps. After irradiation, exponentially growing *M. aeruginosa* was inoculated in the irradiated water to produce an initial cell concentration of about 10^5 cells/ml. Then we monitored the survival of the test organisms in the irradiated water by counting the cells in haemocytometers under microscope.

The culture media for *M. aeruginosa* contains organic matter (N,N-2Bis(2-hydroxymethyl) glycine, $C_6H_{13}NO_4$), metals (Fe, Mn, Al) and inorganic nutrients (N, P).

2.4 Residual effect on *E. coli*

In case of *E. Coli.*, K12 A/λ (F^+), three different combinations of synthetic water were used – (i) Synthetic water containing organic matter (N,N-2Bis(2-hydroxymethyl) glycine, $C_6H_{13}NO_4$), inorganic nutrients (N,P) and metal (0.12 mg/l $FeCl_3,6H_2O$) (ii) Synthetic water containing only metals and inorganic nutrients (no organic matter) (iii) Synthetic water containing only organic matter and inorganic nutrients. After UV-radiation of 400, 800 and 1600 mWs/cm^2, samples were inoculated with *E. Coli.* Cell densities were then monitored for 48 hours.

2.5 Role of H_2O_2 in producing the residual effect

Measurement of H_2O_2, produced due to UV-radiation. H_2O_2, produced during irradiation, was measured using an inverse-fluorometric method [12]. This method involves the transformation of scopoletin (7-hydroxy-6-methoxy—2H-benzopyran-2-one) into a nonfluorescent, colorless product when it reacts with hydrogen peroxide under the action of horseradish peroxidase. The hydrogen peroxide in the sample oxidizes the scopoletin upon addition of horseradish peroxidase, resulting in an immediate decrease of fluorescence. The hydrogen peroxide concentration is determined from the difference in fluorescence before and after addition of catalyst. Calibration curves are obtained by recording the decrease in fluorescence upon addition of H_2O_2 stock solution. The concentration of the stock solution was determined periodically by titration with standard $KMnO_4$.

Production and persistence of H_2O_2 produced by UV-radiation. To identify the possible mechanism that causes the residual effect of UV-radiation, we decided to check the role of H_2O_2. 30-ml of culture media was subjected to UV-radiation from two low-pressure mercury lamps to produce UV-doses of 150, 400, 800 and 1600 mWs/cm^2. H_2O_2-production due to these four different doses was then measured by inverse fluorometric method [12]. Some of the samples were stored in screw capped glass tubes and were then kept in the dark at 4°C temperature to determine the half-lives of UV-induced H_2O_2.

3. RESULTS AND DISCUSSIONS

3.1 Effect of direct UV-radiation

The effect of direct UV-radiation on *M. aeruginosa* for three different UV-doses is shown in Figure 1. As shown in the figure, there was no change in cell density immediately after irradiation for any of the three UV-doses. But after 24 hours of incubation under fluorescent light, there was significant difference in cell density between the irradiated samples and the control (no UV-irradiation). And at the end of the third day, the cell densities in all of the irradiated samples were less than 25 percent of that of the control. After the third day, the green color of the cells that were subjected to a UV-dose of 150-mWs/cm^2, gradually started to fade and at the end of seventh day, all of these cells were either completely faded or their cell-walls were broken. But cells that were subjected to lesser UV-doses (37-mWs/cm^2 and 75-mWs/cm^2) continued to survive. But interestingly, the cell densities in these samples remained the same throughout the incubation period, whereas the cell density in the control increased from $5.4*10^4$ cells per ml to $1.0*10^6$ cells per ml within the same period. And

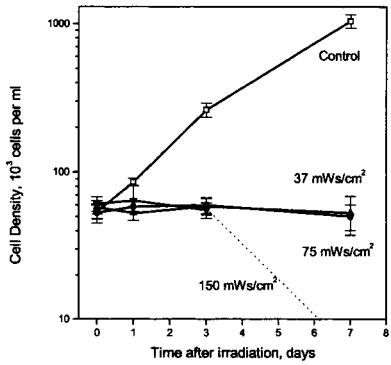

Figure 1. Growth of *M. aeruginosa* after short exposure to germicidal UV-irradiation. The cells were incubated in 60-ml pyrex tube. Vertical bars show standard deviation of each data set. The dotted line indicates that all the cells were dead.

microscopic observation also did not show any cell division in the irradiated samples. This probably indicates damage to the reproduction system of the cells. Interestingly, there was little difference, in terms of cell density, between the effect of 37-mWs/cm^2 dose and 75-mWs/cm^2 dose. But whereas all the cells that was subjected to 75-mWs/cm^2 dose settled to the bottom of the tube, a small number of cells subjected to 37-mWs/cm^2 dose remained in suspension.

3.2 Residual effect on *M. aeruginosa*

A summary of the results of the indirect effects of UV-radiation on algal-culture media containing bicine is detailed in Figure 2 (a) and (b). The figures show the growth profiles of the test organism (*M. aeruginosa*) with time. As shown in the Figure 2(a), when the culture media for *M. aeruginosa* was irradiated by an incident UV-dose of 100-mWs/cm^2, it produced no residual effect on the growth of the test organism. When the dose was increased to 200-mWs/cm^2, initially there was no significant difference between the control and the irradiated sample. But at the end of 7-days, the cell density in the irradiated sample was only 50% of that in the control. When the dose was increased to 400 mWs/cm^2, the residual toxic effect caused complete mortality of all the test organisms. The toxic effect persisted, although some exposed cells survived (after 3-days, cell concentration was only 3% of that in the control) when the irradiated samples were stored for 24-hours before it was inoculated with test organism. But when the storage period was increased to 7-days, no significant residual effect on the growth of test organism was observed for an incident UV-dose of 400-mWs/cm^2 (Figure 2(b)).

But for a higher dose (800-mWs/cm^2) the effect persisted even after 7-days storage (Figure 2(b)). So, for higher UV-dose the residual effect persists for longer duration.

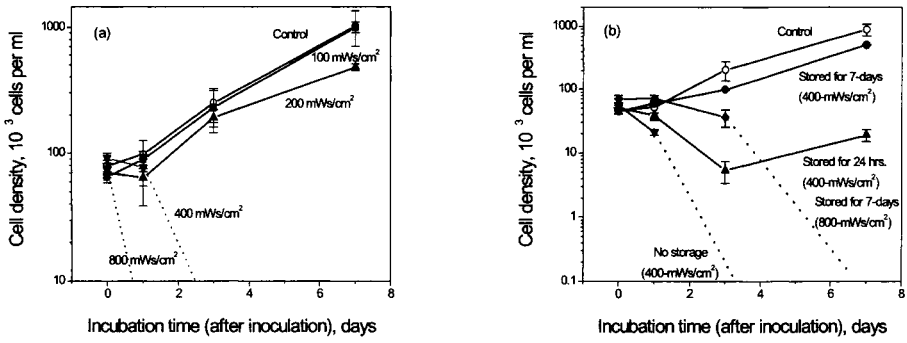

Figure 2. Residual effect of UV-radiation on M. aeruginosa. (a) Test organisms were inoculated in the irradiated water immediately after irradiation . (b) After irradiation, samples were kept in the dark at 4°C for 1-7 days, before the inoculation of test organisms. Vertical bars show the standard deviation of each data set. The dotted lines indicate that all the cells were dead.

The residual effect of UV-radiation can be partly explained by the action of photosensitizers. These are compounds that are transformed from the low-energy ground state to a higher energy electronically excited state by absorption of photon and are capable of transferring their energy to other molecules. The energy transfer may induce chemical reactions producing intermediate compounds like singlet oxygen, hydrogen peroxide and OH-radicals, which in turn may have toxic effect on algae [8,10,13]. Among these species, only H_2O_2 is likely to survive in water for the time period for which the residual effect was observed.

3.3 Residual effect on *E. coli.*

Use of algae as the test organism of biocidal effect allowed the study of residual effect only on specific media; because any variation in the specific media would itself be harmful to algal growth. In an attempt to overcome this problem, we used *E. coli* as the test organism to study the effect of organic matter and metal in producing the residual effect.

Residual effect on media. We found that UV-radiation of algal culture media does not produce any significant residual effect for any of the samples when a contact time of 15 minutes was allowed (Table 1). When the contact time was increased to 90 minutes, 400 and 800 mWs/cm^2 dose did not show any significant residual effect but for the 1600 mWs/cm^2-dose, the population decreased by more than 50%. This indicated the significance of contact time in the residual effect test. So we decided to observe the residual effect for long contact period. As shown in Table 1., after a contact time of 24 hours the percentage reduction (as compared to the control) of bacterial population in all the irradiated samples were more than 97%.

These results indicate that due to UV-radiation some biocidal agent is produced which survives for significant period of time.

Table 1
Residual Effect of UV-radiation on *E. Coli*

UV-dose, mWs/cm^2	Contact time	Percentage reduction	Contact time	Percentage reduction	Contact time	Percentage reduction	Contact time	Percentage reduction
400	15 min	N.R.*	90 min	24.5%	24 hrs	97.3%	48 hrs	99.4%
800	15 min	N.R.	90 min	22.5%	24 hrs	98.6%	48 hrs	100%
1600	15 min	N.R.	90 min	59.5%	24 hrs	100%	48 hrs	100%

*N.R. indicates no reduction as compared to the control

Effect of organic matter and metal. When synthetic water containing only metal and inorganic nutrients were irradiated under UV-light, no significant residual effect of UV-radiation was evident on *E. coli.*, even after 48 hours. This clearly indicates that the presence of organic matter is essential for the residual effect.

Irradiated synthetic water containing only bicine and nutrients (no metal) showed very little residual effect. After 24 hours, the cell densities in the irradiated samples were considerably low. But during incubation, the cell densities continued to increase in the control as well as in the irradiated samples. This indicates that metals plays an important role in producing the residual effect.

3.4 Production of H_2O_2 due to UV-radiation

H_2O_2 production in UV-irradiated culture media. A UV-dose of 150 mWs/cm^2 produced about 0.54 μM H_2O_2. Higher UV-dose produced higher concentration of H_2O_2. When the H_2O_2 concentrations were plotted against the UV-doses, it produced a good linear fit (r^2 = 0.994)(Figure 3).

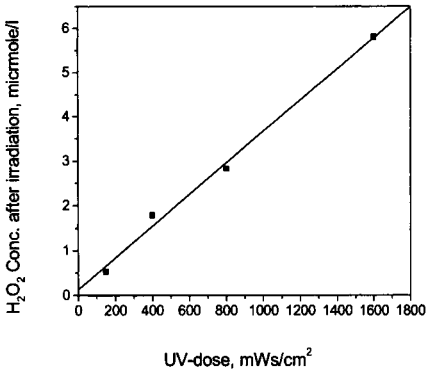

Figure 3. H_2O_2 generation due to UV-irradiation. The linear fit is described by the equation, y = 0.1322 + 0.00354 x (r^2 = 0.99).

These results indicate a very good correlation between the residual effect of UV-radiation and H_2O_2 production. As was shown in Figure 2(a), increasing the UV-dose also increased the extent of residual effect. In agreement with this trend, increased UV-dose also produced increasing H_2O_2. This suggests that H_2O_2 could indeed play a key role in producing the residual effect.

H_2O_2 production in UV-irradiated synthetic water. We showed that various combinations of organic matter, metals and nutrients produced different extent of residual effect. If H_2O_2 was mainly responsible for the residual effect, than it could be expected that H_2O_2 production in those synthetic waters would show similar pattern as that of residual effect. To verify this, different combination of bicine, metal and nutrients were irradiated by UV light and then H_2O_2 production was measured. The results are shown in Table 2.

No H_2O_2 was produced in synthetic water containing no organic matter. For similar UV-dose, synthetic water containing only organic matter and inorganic nutrients produced the least amount of H_2O_2. And synthetic water containing iron and organic matter produced the highest concentration of H_2O_2. These results are also consistent with the previous results- UV-irradiation of synthetic water with only nutrients and metal did not produce any residual effect; metal free synthetic water only produced little residual effect; and combination of metals, nutrients and bicine produced the maximum residual effect. So, there is clear correlation between the H_2O_2 production and residual effect.

3.5 Persistence of H_2O_2 in UV-irradiated water

The results are shown in Figure 4. As shown in the figure, H_2O_2-produced due to UV-radiation can persist in the water for long period. While stored in the dark at 4°C, UV-produced H_2O_2 showed a first order decay with half-life of about 7-days. This is not consistent with the fact that the residual effect of UV-radiation diminishes at a faster rate. Because if H_2O_2 alone was responsible for producing the residual effect, then it would have continued for a longer duration

Moreover, when H_2O_2 was externally added into non-irradiated culture media, there was almost no change in H_2O_2 concentration during the 7-days of storage. This result indicate that the change in H_2O_2 concentration in the irradiated sample was probably due to reaction with other reactive species.

Table 2
H_2O_2 production in UV-irradiated synthetic water

Constituents	UV-dose, mWs/cm^2	H_2O_2 Concentration μ-mole per liter
$FeCl_3,6H_2O$, Nutrient	1600	0
Bicine (500 mg/l), Nutrient	1600	2.09 ± 0.12
Bicine (500 mg/l), Nutrient, $FeCl_3,6H_2O$ (0.12 mg/l)	1600	7.09 ± 0.47

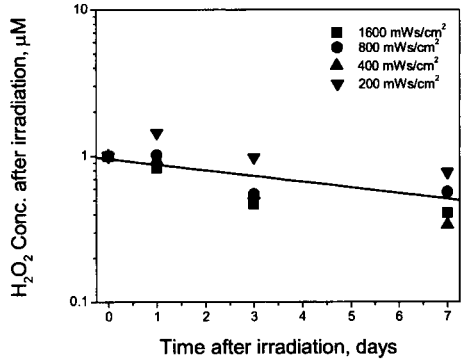

Figure 4. Decay of UV-irradiation-induced H_2O_2 with time. The best fit curve is drawn for the data of 800-mWs/cm^2 UV-dose.

4. CONCLUSIONS

This study shows that UV-radiation has the potential to be used as an effective means to control algae. As relatively small UV-dose (37 mWs/cm^2) can control the algal reproduction for a significant period of time, UV-radiation prior to the bloom period may act as an effective means to control seasonal algal bloom. Our study clearly shows that UV-radiation may produce residual effect that is harmful to microorganisms (*M. aeruginosa* and *E. Coli.*). It was also shown that the residual effect might persist for long duration. The extent of residual effect increased with increasing UV-dose. However, residual effect of UV-radiation is highly dependent on the constituents of the irradiated water. Presence of organic matter which can act as photosensitizer is essential for the residual effect of UV-radiation. UV-radiation produced µM level of H_2O_2 in the irradiated water and the H_2O_2 production increased with increasing UV-dose. The H_2O_2, that was produced due UV-irradiation showed a first order decay (half-life of about 7-days) while stored in the dark at 4^{0}C. But when H_2O_2 was externally added to the same synthetic water, there was almost no change in H_2O_2 concentration during 7-days of storage under the same conditions. These results suggest the presence of reactive species which react with H_2O_2 in the irradiated water. The extent of residual effect as well as the H_2O_2 production increased in the presence of metal ions (Fe^{3+}). Hence we concluded that both H_2O_2 and metal ions play significant role in producing the residual effect of UV-radiation.

REFERENCES

1. Groocock, N.H. (1984) Disinfection of drinking water by ultraviolet light. Jour. Institution of Water Engrs. Scientists, 38(2): 687-689.

2. Chang, J. C. H., Ossoff, S. F., Lobe, D. C., Dorfman, M. H., Dumais, C. M., Qualls, R. G. and Johnson, J. D. (1985) UV-inactivation of pathogenic and indicator microorganisms. Appl. Environ. Microbiol., 49: 1361-1365.
3. Kamiko N, Ohgaki S (1989) RNA coliphage Qb as a bioindicator of the UV disinfection efficiency. Water Science And Technology 21(3): 227-232.
4. Karanis P, Maier-W-A, Seitz-H-M, Schoenen-D (1992) UV sensitivity of protozoan parasites. AQUA (OXFORD) ,41(2): 95-10
5. Battigelli, D. A., Sobsey, M. D., and Lobe, D.C. (1993) The inactivation of Hepatitis A virus and other model viruses by UV irradiation. Wat. Sci. Technol., 27(3-4): 339-342.
6. McKnight, D. M., Chisholm, S. W., and Harlemann, D. F. (1983) $CuSO_4$ treatment of nuisance algal blooms in drinking water reservoirs. Environ. Manage. 7, 311-320.
7. Graham, N. J. D., Wardlaw, V. E., Perry, R and Jiang, J. Q. (1997) The significance of algae as trihalomethane precursors. Proc. Of IAWQ-IWSA joint conference on Reservoir management and water supply. Prague, Czech Republic, pp. 93-100.
8. Gjessing E. T. and Källqvist T. (1991) Algicidal and chemical effect of UV-radiation of water containing humic substances. Wat. Res. 25: 491-494.
9. Alam, M. Z. B., Otaki, M., Furumai, H., and Ohgaki, S. (1998) Control of Algal growth by UV-radiation. Environmental Engineering Research, JSCE. 35, 111-118.
10. Lund V. and Hongve D. (1994) Ultraviolet irradiated water containing humic substances inhibit bacterial metabolism. Wat. Res. 28(5), 1111-1116.
11. Japan Water Works Association (1993), Standard methods for examination of water.
12. Holm,T. R, George, G. K., Barcelona, M. J. (1987) Fluorometric determination of hydrogen peroxide in ground water. Anal. Chem., 59, 582-586.
13 Hessen D.G. and Donk E.V. (1994), Effects of UV-radiation of humic water on primary and secondary production. Water, Air and Soil Pollution. 75: 325-338.

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Bacteriophages, coliform and fecal coliform bacteria in wastewater in southern Thailand

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The study of microbial communities in wastewater has generally focused on water pollution and water quality management issues. Coliform bacteria and also bacteriophages have been recognized as having potential for use as biological indices for contamination of water. This study was conducted to confirm the presence of various types of bacteriophages, coliform and fecal coliform bacteria in raw wastewater and treated wastewater from 15 sources, including domestic, industrial and agricultural wastewaters. Bacteriophages investigated in this study were bacteriophage B, bacteriophage C and F-specific bacteriophages. The results showed that F-specific bacteriophages, in particular RNA–F-specific bacteriophage were present in sewerage wastewater and wastewater from slaughterhouses, pig farms and cow farms. Bacteriophage C was detected at higher levels than bacteriophage B and F–specific bacteriophages in almost all the wastewater sources examined. Correlation between the concentration of each bacteriophage and coliforms/fecal coliforms was statistically calculated using linear correlation analysis. It can be concluded that bacteriophage B, bacteriophage C, F specific bacteriophage and RNA-F-specific bacteriophage can be used as pollution indicators while F specific bacteriophage and RNA-F-specific bacteriophage can be used as indicators of animal waste pollution.

1. INTRODUCTION

A variety of microorganisms are found in wastewater. However, as far as engineers and water quality microbiologists are concerned, the agents of greatest interest are coliform becteria and bacteriophages. There are two principal groups of coliform bacteria: the fecal coliforms (of which the bacterium *Escherichia coli* has been most studied) and the total coliform group, which includes the fecal coliforms and consists mainly of species from the genera *Citrobacter*, *Enterobacter*, *Escherichia*, and *Klebsiella*. The former are exclusively fecal in origin, whereas the latter, although commonly found in feces, also occur naturally in soils and waters. Only the fecal coliforms are definitive indicators of fecal pollution. In water bacteriology the total coliforms are regarded as "presumptive" indicators of pollution but in wastewater bacteriology, this group is of considerably less importance, because many are non-fecal in origin and because they can multiply in the environment under suitable conditions, especially in hot climates. Thus their presence or numbers may not necessarily relate to either the occurrence or degree of fecal pollution [1].

Bacteria are host to a special group of viruses called "bacteriophages" or "phages". Bacteriophages have been recorded for many species of bacteria. Coliphages were recognized as bacteriophages that infect and replicate in coliform bacteria and appear to be present wherever bacteria from the total and fecal coliform groups are found. However, there is little literature on the relationship between coliforms and bacteriophages in the aqueous environment, especially in tropical areas. Further knowledge on the presence of coliforms and bacteriophages in various types of raw wastewater, treated wastewater and natural water together with the correlation between bacteriophages and coliforms would be highly valuable in helping to assess the quality of water and wastewater. This study was carried out to investigate the presence of these groups of microorganism in various sources of raw wastewater, treated wastewater and natural water. Results of the study provide information on the relationship between coliforms and bacteriophages in the aqueous environment and their use as indicators of water quality.

2. METHODOLOGY

2.1. Wastewater and water sampling

Grab samples of wastewater and water from natural sources were taken from 22 points during July-September 1998 in Songkhla province in southern Thailand. At each sampling point, wastewater and water samples were taken 3 times. A total of 66 wastewater and water samples were taken from 2 sources of natural water, 14 sources of raw wastewater and 6 sources of treated wastewater. Natural water samples were taken from river and coastal water. The 14 raw wastewater samples were taken from 4 domestic sources, 6 sources of agro-based industries and 4 agriculture/aquaculture sources, while the treated wastewater samples were taken from 3 domestic sources and 3 sources from agro-based industries. Table 1 gives full details of water and wastewater samples taken during this study. All samples were kept in an ice box, transferred to the laboratory and analysed immediately upon arrival.

2.2. Analysis of physical and chemical characteristics

pH, temperature, suspended solids (SS), chloride, and chemical oxygen demand (COD) were determined for all samples. Physical characteristics were determined at the sampling site. Chloride, SS and COD were analyzed following the procedures described by APHA, AWWA & WEF, (1995) [2].

2.3. Analysis of biological characteristics

(a) Enumeration of coliform bacteria and fecal coliform bacteria

Total coliform bacteria and fecal coliform bacteria populations in the samples were determined by using the multiple tube fermentation technique as described by APHA, AWWA & WEF, (1995) [2].

(b) Coliphage assay

Water and wastewater samples were pretreated to remove some of the bacteria present by centrifuging at 12,000 rpm for 20 minutes at 4 °C. Indigeneous coliphage enumeration was performed following the method described by Ketratanakul in 1989 [3]. The double agar layer technique was done with media containing 1% polypeptone, 0.5% yeast extract (Difco), 0.5% NaCl, 0.02% $MgSO_4$. $7H_2O$, 0.005% $MnSO_4.4H_2O$ and 0.15% dextrose with the final pH adjusted to 7.0±0.2. This media was used for culturing host bacteria, dilution and as a base medium for bottom agar and top agar. Bottom agar and top agar consisted of 1.1% and 0.6% bacto agar (Difco) respectively. The host bacteria used in this investigation were young

Table 1
Water and wastewater sampling points and sample numbers

Sampling points	Number of Sample		Total
	Raw wastewater	Treated wastewater	samples
1. Domestic sources			
Hospital	3	3	6
Residential building	3	-	3
Hotel	3	3	6
Sewerage	3	-	3
Septic tank	-	3	3
2. Agro-based industry			
Frozen sea food industry	3	3	6
Fish mill industry	3	3	6
Fishery port	3	-	3
Slaughterhouse (pig)	3	3	6
Slaughterhouse (cattle)	3	-	3
Slaughterhouse (chicken)	3	-	3
3. Agricultural areas			
Pig farm	3	-	3
Cow farm	3	-	3
Shrimp farm	3	-	3
Fish farm	3	-	3
4. Natural waters			
River water	3	-	3
Coastal water	3	-	3

E. coli B, *E. coli* C and *E. coli* K12 F^+ (A/λ) with a cell age of 3.5-4 hours and a cell count of about 10^8 CFU/mL.

A sample or a diluted sample (0.1 mL) was mixed with 0.3-0.35 mL of young bacterial host culture, 0.25 mL of 0.1 M $CaCl_2$ and 4.0 mL of melted top agar. This mixture was poured over the surface of bottom agar, allowed to solidify and incubated at 37°C for 12-16 hours. After incubation, plaques were counted. Coliphage B, coliphage C and F-specific coliphage were assigned by the use of bacterial hosts *E. coli* B, *E. coli* C and *E.coli* K12 F^+ (A/λ) respectively. RNA-F-specific coliphage was enumerated separately by plating with and without RNase A, Sigma (100 μg/plate) on a lawn of *E.coli* K12 F^+ (A/λ) bacterial host. The difference in counts on plates with and without RNase showed the number of RNA-F-specific coliphages present.

2.4. Statistical analysis

Statistical analysis was performed using linear regression analysis to investigate correlation between each bacteriophage with total coliform bacteria and fecal coliform bacteria in water and wastewater samples.

3. RESULTS AND DISCUSSION

3.1. Physical and chemical characteristics of water and wastewater samples

The chemical and physical characteristics of water and wastewater samples investigated in this study are summarized in Table 2. Sample temperature ranged from 18 to 36°C and pH ranged from 6.61 to 10.00. The temperature of the wastewater sample taken from a frozen sea food industry sampling site was found to be lowest with a value of 18°C. Chloride concentrations of samples were in the range 11-18,443 mg/L, with higher concentrations attributed to the influence of salts contained in the samples. Samples from a fishery port, fish farm, and shrimp farm contained chloride concentrations higher than 10,000 mg/L. Suspended solids and COD were between 8.6-2,560 mg/L and 10-4,028 mg/L, respectively. The highest COD concentration was determined in samples taken from a pig farm while the lowest COD concentration was found in river water samples.

3.2. Appearance of total coliform bacteria and fecal coliform bacteria in the samples

Figure 1 shows the concentrations of total coliform bacteria and fecal coliform bacteria detected in water and wastewater samples. Total coliform bacteria and fecal coliform bacteria in raw wastewater taken from domestic sources were found to be in the range $4.3 \times 10^6 - 1.1 \times 10^8$ MPN/100mL, and $2.3 \times 10^6 - 2.4 \times 10^7$ MPN/100mL respectively. Hospital wastewater samples contained the highest concentrations of total coliform bacteria and fecal coliform bacteria within this set. After wastewater was treated, concentrations of total coliform and fecal coliform bacteria were found to decrease by more than 95%. Total coliform bacteria and fecal coliform bacteria in samples from agro-based industrial sources were found to be greater than 10^4 MPN/100mL in all cases. Total coliform and fecal coliform bacteria from slaughterhouse samples were found to be highest within the agro-industry set with a concentration of more than 10^7 MPN/100mL.

Table 2
Summary of physical and chemical characteristics of water and wastewater samples

Sampling points	Temperature (°C)	pH	Chloride (mg/L)	Suspended Solids (mg/L)	COD (mg/L)
1. Domestic sources					
Raw wastewater	26-31	7.15-10.00	39-242	17-164	78-465
Treated wastewater	29-31	7.25-7.92	65-130	12-72	59-416
2. Agro-based industry					
Raw wastewater	18-36	6.45-8.99	30-11,496	40-1,680	298-3,167
Treated wastewater	28-34	6.61-9.42	95-1,795	12-160	172-417
3.Agricultural areas					
Raw wastewater	27-32	7.05-8.49	50-15,704	14-2,560	791-4,028
4. Natural waters					
River water	29-30	6.80-6.90	11-16	38-60	10-40
Coastal water	29-31	8.08-8.10	18,002-18,443	8.6-10.2	-

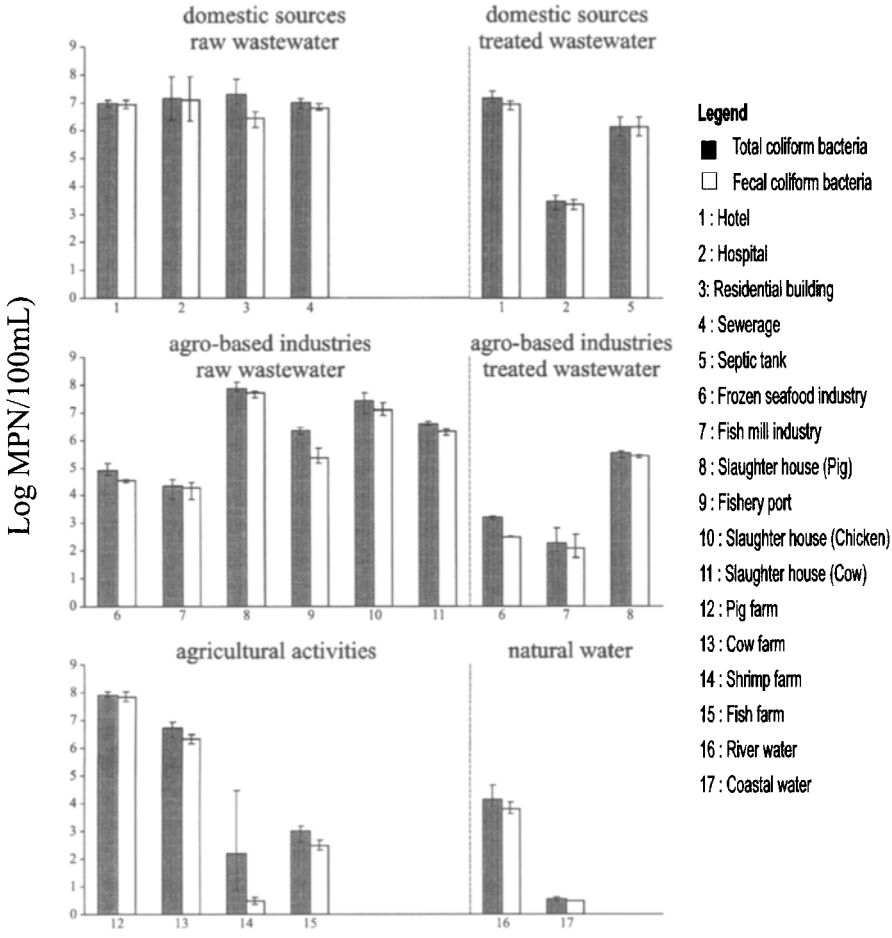

Figure 1. Numbers of total coliform bacteria and fecal coliform bacteria in samples.

In samples from agricultural areas, concentrations of total coliform bacteria and fecal coliform bacteria were found to be within the range from < 3 - 1.1 x 10^8 MPN/100mL. Within this set, the highest concentrations of both bacterial groups were observed in samples from a pig farm, whereas the lowest concentrations were found in sample from a shrimp farm. Total coliform bacteria and fecal coliform bacteria of shrimp farm samples ranged between <3 - 43 MPN/100mL and <3 – 7 MPN/100mL respectively. Samples from the fish farm were found to contain coliform and fecal bacteria levels higher than samples from the shrimp farm. Total coliform and fecal coliform bacteria in coastal water samples were detected to be lower than samples from river water samples. However, concentrations of total coliform and fecal coliform bacteria detected in river water samples were observed to be three times lower than concentrations detected in wastewater samples from sewerage.

3.3. Appearance of bacteriophages in the samples

Coliphage B, coliphage C, F-specific coliphage and RNA-F-specific coliphage were detected in all samples as illustrated in Figure 2. It was observed that coliphage C was detected in highest quantities in almost samples examined in this study. Among the raw wastewater samples from domestic sources and agro-based industry, it was found that bacteriophages of all types were detected to be lowest in samples from fish mill industry and highest in samples form the pig slaughterhouse. However, the highest concentration of all groups of bacteriophages was determined in wastewater samples from the pig farm, with a value of more than 10^4 PFU/mL. Bacteriophages were not detected in the samples from shrimp and fish farms or samples from coastal water. Bacteriophages were detected in river water samples, but the concentrations were very low.

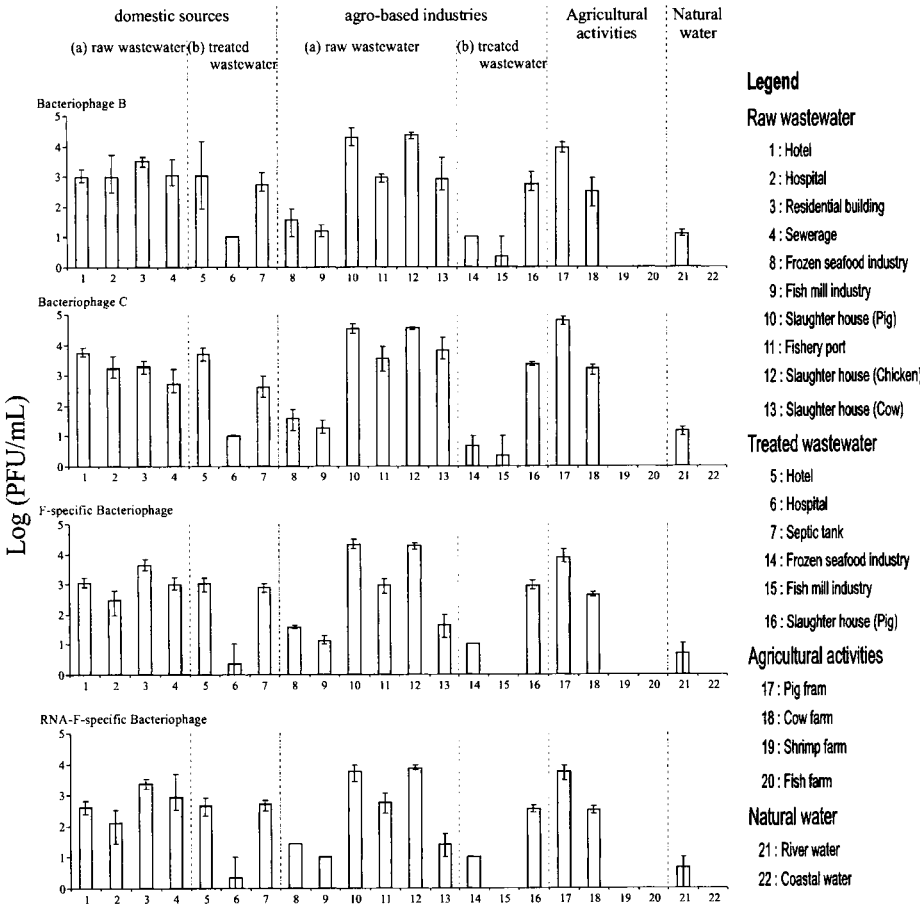

Figure 2. Concentrations of bacteriophages detected in water and wastewater samples.

Coliphage B, F-specific coilphage and RNA-F-specific coliphage were determined at highest levels in samples from a plant for killing chickens, with average concentrations of 2.23 x 10^4 PFU/mL, 2.07 x 10^4 PFU/mL and 6.99 x 10^3 PFU/mL respectively. Coliphage C was detected in highest quantities in samples from the pig farm, with an average value of 5.26 x 10^4 PFU/mL.

For raw wastewater samples taken from domestic sources, 4 groups of bacteriophages were detected with in the range of 10^2-10^3 PFU/mL. Effluent samples from the septic tank also showed four groups of bacteriophages with an average concentration range of 4.95 x 10^2 to 6.59 x 10^2 PFU/mL. In this investigation, RNA-F-specific coliphage was determined to be present at the level of 10^2 to 10^3 PFU/mL in all raw wastewater samples taken from domestic sources. Other studies have found RNA-F-specific coliphage concentration levels in raw sewage ranging from 10^2 to 10^5 PFU/mL by utilizing male strain of *Escherichia coli* or *Salmonella typhimurium* strains as specific host bacteria [3-6]. In 1990, Havelaar *et al.*[7] also reported that RNA-F-specific coliphages could be often detected in faeces from pigs and adult chickens. Moreover, RNA-F-specific coliphage of all samples investigated in this study were in the range of 23-100% of indigeneous coliphages (Table 3). This finding is consistent with Ketratanakul and Ohgaki's study in 1988 [8]. They reported that the percentage of RNA-F-specific coliphage detected was found to be 19-83% of indigeneous coliphage content detected in sewage samples in Japan. It can therefore be concluded that the appearance of RNA-F-specific coliphage in wastewater samples in this study is similar to patterns previously reported around the world.

3.4. Correlation between bacteriophages, total coliform bacteria and fecal coliform bacteria

Correlation coefficients (r) for the relationships between numbers of bacteriophages, total coliform bacteria and fecal coliform bacteria are summarized in Table 4. The coefficient for the relationship between numbers of coliphage B, coliphage C, F-specific coliphage, RNA-F specific coliphage and the fecal coliform bacteria numbers over all samples was observed to

Table 3
The average percentage of RNA-F-specific coliphages in wastewater samples.

Wastewater Source	Percentage in Raw Wastewater	Percentage in Treated Wastewater
Hospital	49	76
Hotel	30	36
Residential Building	80	-
Sewerage	78	-
Septic Tank	-	78
Fish Mill Industry	100	-
Frozen Sea Food Industry	78	94
Slaughterhouse: Pigs	72	39
Fishery Port	23	-
Slaughterhouse: Chicken	36	-
Slaughterhouse : Cattle	60	-
Pig Farm	40	-
Cow Farm	73	-

Table 4
Correlation coefficients (r) for the relationships between number of bacteriophages, total coliform bacteria and fecal coliform bacteria.

Bacteriophages	Correlation Coefficients (r)					
	A		B		C	
	Total Coliform Bacteria	Fecal Coliform Bacteria	Total Coliform Bacteria	Fecal Coliform Bacteria	Total Coliform Bacteria	Fecal Coliform Bacteria
Coliphage B	0.902	0.906	0.810	0.780	0.660	0.720
Coliphage C	0.899	0.907	0.780	0.770	0.740	0.750
F-specific Coliphage	0.886	0.890	0.820	0.790	0.740	0.770
RNA-F-specific Coliphage	0.883	0.885	0.810	0.770	0.730	0.770
Number of Samples	198	198	126	126	45	45

A: Data from all samples was used in calculation.
B: Only data from raw wastewater samples was used in calculation.
C: Only data for raw wastewater from slaughterhouses and animal farms was used in calculation.

be high with a value of about 0.9. This implies that four types of coliphages could be used as a pollution indicator index. However, meaningful relationships between F-specific coliphage or RNA-F-specific coliphage and total coliform and fecal coliform bacteria could also be observed. When wastewater data of slaughterhouses and animal farms were used for correlation analysis, the r values of the data set between fecal coliform bacteria and either F-specific coliphage or RNA-F-specific coliphage were observed to be highest. This indicates that RNA-F-specific coliphage and F-specific coliphage have potential for use as an indicator index for animal waste pollution.

4. CONCLUSION

From the results obtained in this study, the following conclusions can be drawn.

1) When *E. coli* K12, F^+ (A/λ) is used as a bacterial host, RNA-F-specific coliphages in raw wastewater samples from domestic sources can be consistently detected at numbers more than 10^2 PFU/mL. 23-100% of indigeneous coliphages in wastewater from domestic sources, agricultural activities and agro-based industry were found to be RNA-F-specific coliphages.

2) Based on the quantitative data on bacteriophage counts and correlation analysis between numbers of total coliform bacteria, fecal coliform bacteria, and each type of bacteriophage, it can be concluded that bacteriophage B, bacteriophage C, F specific bacteriophage and RNA-F-specific bacteriophage can be used as pollution indicators while F specific bacteriophage and RNA-F-specific bacteriophage can be used as indicators of animal waste pollution.

REFERENCES

1. R.G. Feachem, D. J. Bradley, H. Garelick and D. D. Mara, Sanitation and Disease, World Bank Studies in Water Supply and Sanitation 3 (1983).
2. APHA, AWWA & WEF, Standard Methods for the Examination of Water and Wastewater, 19th edition, American Public Health Association, Washington, D.C. (1995).
3. A. Ketratanakul, Evaluation of Coliphages as Model Viruses in Water and Wastewater Environment, Doctoral Dissertation, University of Tokyo, Tokyo, Japan (1989).
4. K. Furuse, A. Ando, S. Osawa and I. Watanabe, Appl. Environ. Microbiol, 41: 1139-1143 (1981).
5. A.H. Havelaar and W.M. Hogoboom, Proceedings of International conference on water and wastewater microbiology, Newport Beach, California, USA, (1988).
6. S. Danteravanich, Application of Polymerase Chain Reaction for Health Related Viral Indicators Detection in Wastewater, Ph.D. Dissertation, University of Tokyo, Tokyo, Japan, (1992).
7. A.H. Havelaar, W.M. Pot-Hogeboom, K. Furuse, R. Pot, and M.P. Hormann, Journal of Applied Bacteriology, 69:30-37 (1990).
8. A. Ketratanakul and S. Ohgaki, Indigeneous, Manuscript for Health - Related Water Microbiology Seminar at IAWPRC 14th Biennial Conference, Brighton, (1988).

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Assessment of treatment efficiency by quantitative recovery of indicator bacteria and pathogens in sewage effluents.

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The present study delineates into quantification and identification of bacterial indicators and enteropathogens in sewage effluents and their maximal recovery by modifying conventional enrichment media. The study also examined the drug sensitivity of the isolates and virulence property of *E. coli.* Five commonly used selective culture media were employed for the quantitative isolation of the indicator bacteria and/or enteropathogens including the non-selective Nutrient Agar for total aerobic heterotrophic count, also a pollution indicator. The organisms isolated were the species of *Aeromonas, Bacillus, Citrobacter, Escherichia, Enterobacter, Klebsiella, Pasturella, Proteus* and *Pseudomonas*. The total coliform count at the discharge point of the sewage treatment plant was 5.3×10^5 cfu/100ml, whereas it was 1.4×10^5 cfu/100ml in the receiving river water. The faecal coliform count at the discharge point of the sewage treatment plant and in the water of the river Buriganga was respectively 8.4×10^4 cfu/100ml and 4.4×10^4 cfu/100ml, whereas the number of total heterotrophs were respectively 2.8×10^7 cfu/100ml and 5.5×10^6 cfu/100ml. Considerable recovery of stressed/injured cells was evident when the newly designed pre-enrichment and enrichment media were used as compared to the conventional enrichment medium. Almost 90% of the isolates was resistant to bacitracin, whereas 96%, 90%, 80% and 73% of the isolates were susceptible to gentamycin, kanamycin, ciprofloxacin and amoxycillin respectively. On haemagglutination test, all the five *E.coli* isolates were found to be nonhaemolytic as well as nonenteropathogenic.

1. INTRODUCTION

Pollution in its broadest sense includes all changes that curtail natural utility and exert deleterious effect on life. The crisis triggered by the rapidly growing population and industrialization with the resultant degradation of the environment causes a grave threat to the quality of life. Degradation of water quality is the unfavourable alteration of the physical, chemical and biological properties of water that prevents domestic, commercial, industrial, agricultural, recreational and other beneficial uses of water [1]. Sewage and sewage effluents are the major sources of water pollution. Sewage is mainly composed of human faecal material, domestic wastes including wash-water and industrial wastes. If these are discharged into river or sea without proper treatment, this may cause threat to the aquatic life and other consumers [2]. Unfortunately there is no standard or effective sewage treatment plant in Bangladesh. Everyday a large volume of sewage is being discharged into various rivers without proper treatment [3].The existing lagoon type sewage treatment plant designed for a

peak sewage flow of 40 mgd [1] is not adequate for the treatment of Dhaka city sewage to a satisfactory level.

Water related diseases remain the major cause of mortality and morbidity in Bangladesh [4]. More than 50 percent of all illnesses in Bangladesh are waterborne or water-related [5]. About 15 percent of cases of death among 5-14 year old children are caused by diarrhoea. Enteropathogens and other non-indigenous bacterial species when discharged into natural environments have a tendency to decline [6]. There are also reports that *Escherichia coli* , *Klebsiella* spp. , *Vibrio* spp. and other enteropathogens may survive indefinitely in tropical waters and may even multiply [7,8]. Non-indigenous organisms in the environment are exposed to various types of sublethal stress, starvation and injured and could be in a state of metabolic arrest [9,10]. Most recovery or enrichment media are designed for clinical purposes and contain excessive nutrients and toxic selective agents which may have an inhibitory effect on bacterial cells of environmental origin and may adversely affect their recovery. Pre-enrichment or enrichments with growth stimulating and cell resuscitating components have been designed to repair and recover stressed/injured bacterial cells with some success [9-11].

Waterborne diseases are often treated with various antibiotics. However, pathogens which are not inherently resistant to an antibiotic may become resistant to that antibiotic by mutation or by acquiring a drug resistant plasmid. In the presence of the drug, the mutated pathogen has a selective advantage and may replace the parent type [12]. Thus drug resistant pathogens in the environment might be capable of producing more severe outbreak of epidemics. With this end in view, the specific objective of this investigation has been designed to isolate and identify the indicators and/or enteropathogens in sewage effluents and their quantification in effluents and discharged river water. The newly designed pre-enrichment and enrichment techniques were used for maximal recovery of indicators and enteropathogens from the environmental samples. This study also includes testing of drug sensitivity of the organisms and the virulence property of the *E. coli* isolates.

2. MATERIALS AND METHODS

2.1 Sampling site and collection of sample

Forty-two samples of sewage effluents and river water were collected from the lagoon system of sewage treatment plant at Pagla, situated on the northern side of Dhaka-Narayanganj road. The plant receives the sewage and after partial physical and natural biological treatment in the lagoon, the effluent is discharged into the river Buriganga. Six sites in the lagoon and one at the river Buriganga were chosen for the collection of samples. These sites include, inlet (sampling site 1,where the sewage effluents from the pipeline enter the plant), measuring point (sampling site 2, where liquid is collected after partial sedimentation of solids), oxidation pond No. 5 (sampling site 3), oxidation pond No. 10 (sampling site 4) and oxidation pond No. 15 (sampling site 5) (out of twenty oxidation lagoons, three were chosen for the collection of samples), discharge point (sampling site 6) and water from the downstream of the river Buriganga (sampling site 7).

Samples were collected monthly in pre-sterile screw capped 500- ml glass bottles during June-November 1997. Within one hour of collection, samples were carried to the laboratory

in an insulated icebox. Microbiological examinations with the samples were carried out as promptly as possible after collection or were stored at 4^0 C in a refrigerator until use.

2.2 Isolation of indicators and pathogens

Five selective media such as MacConkey agar (MAC), Eosin Methylene Blue agar (EMB), Xylose Lysine Deoxycholate agar (XLD), Deoxycholate Citrate agar (DCA), mFC agar and the non- selective Nutrient agar (NA) were used for the isolation and quantification of the indicators and enteropathogens.

The prevalence of the indicator bacteria such as Total coliform (TC), Faecal coliform (FC), and Total aerobic heterotrophs (TH) has been quantified to assess treatment efficiency. Attempts have also been taken to isolate and identify pathogenic bacteria prevalent in the wastewater and river water.

All the media were sterilized by autoclaving at 121^0C for 15 minutes unless otherwise specified. Spread plate technique was used for isolation and quantification of organisms following ten-fold serial dilution of the sample. The inoculated plates were normally incubated at 37^0C for indicators and pathogens except for faecal coliforms which were incubated at 44 ± 0.5^0C. Incubation period was usually 24 to 48 h. The counts were reported as 'colony forming unit' per ml or 100 ml.

2.3 Pre-enrichment and enrichment approaches

A number of pre-enrichment and enrichment media were designed and used simultaneously with the conventional enrichment [13] medium to compare the relative efficiency of the newly designed enrichment media. The pH of all the pre-enrichment and enrichment media was adjusted to 7.0 ± 0.2 before sterilization. The compositions of the pre-enrichment (PE) and enrichment media are as follows:

Composition of the PE-1 (g/l)

Protease peptone 5.0, yeast extract 2.0, K_2HPO_4 2.0, KH_2PO_4 1.0, NaCl 5.0, Na-pyruvate 0.5, distilled water 1l. An aliquot of 10ml of sample was inoculated in 40ml sterile PE-1 liquid medium and was incubated for 6 h at 37^0C.

Composition of the PE-2 (g/l)

Glucose 5.0, protease peptone 2.0, yeast extract 1.0, K_2HPO_4 2.0, KH_2PO_4 1.0, NaCl 3.0, bile salts 1.0, distilled water 1l. An aliquot of 10ml from inoculated pre-enrichment medium-1 was dispensed in 40ml of sterile pre-enrichment medium-2 and was incubated for 4 h at 37^0C.

Composition of the newly designed enrichment medium (g/l)

Ingredients of PE-2 + sodium deoxycholate 0.5, brilliant green 0.03, neutral red 0.02. An aliquot of 10 ml from PE-2 was dispensed in 40ml of enrichment broth and was incubated at 37^0C for 15-16 h.

Ten ml each of all the samples was inoculated into 40ml of Hajna [13] broth and incubated for 15-16 h at 37^0C to assess relative recovery potential and was compared with the newly designed pre-enrichment and enrichment media for isolation and quantification of indicators

and pathogens as described in APHA (1992) [14]. The isolates were identified following the methods as described in Manual of methods for General Bacteriology [15].

2.4 Drug sensitivity of the isolates

All the thirty isolates were subjected to drug sensitivity tests using antibiotic discs of ampicilin (AMP, 10 μg), amoxycilin (AMC, 30 μg), bacitracin (BA, 10 μg), ciprofloxacin (CIP, 5 μg) doxycyclin (DO, 30 μg), gentamycin (GM, 10 μg), kanamycin (K, 30 μg), nalidixic acid (NA, 30 μg) and tetracycline (TE, 30 μg).

2.5 Virulence properties of *E. coli* isolates

The haemolytic activity of *Escherichia coli* isolates was examined on blood agar plate containing 5% defibrinated sheep blood. The *Escherichia coli* isolates were tested for serological identification. The three polyvalent antisera used in the examination were: *Escherichia coli* polyvalent 2 [Types 026; k60 (B6), 055; k59 (B5), 0111; k58 (B4), 0119; k69 (B14), 0126; k71 (B 16)], *Escherichia coli* polyvalent 3 [Types 086; k61 (B 7), 0114; k90 (B), 0125; k70 (B15), 0127; k63 (B8), 0128; k67 (B12)], *Escherichia coli* polyvalent 4 [Types 044; k74 (2), 0112; k66 (B11), 0124; k72 (B 17), 0142; k86 (B)].

Haemagglutination reaction was also observed for the isolated *Escherichia coli* cells [16].

3. RESULTS AND DISCUSSION

The number of organisms isolated by routine technique increased many times following the newly designed pre-enrichment and enrichment techniques as compared to the conventional enrichment medium of Hajna [13] particularly when XLD and MacConkey agar were used as isolation media. The average counts on XLD before enrichment, after enrichment with the newly designed media and with Hajna's enrichment were respectively 1.34×10^4 cfu/ml, 2.77×10^7 cfu/ml and 2.56×10^6 cfu/ml while with MacConkey medium the numbers were respectively 1.69×10^3 cfu/ml, 2.6×10^7 cfu/ml and 1.1×10^7 cfu/ml (Figure 1).

Conventional enrichment and isolation media contain luxury concentrations of carbon, nutrients and selective agents like bile salts, deoxycholic acid, other toxic selective agents including dyes. These selective ingredients exert an adverse influence in the repair and /or resuscitation of the injured cells. Pre-enrichment with growth stimulating substances like sodium pyruvate and yeast extract, minus toxic selective agents, was designed to repair and resuscitate the stressed/injured cells. Addition of such substances in the enrichment medium with low but adequate selective agents have resulted in increased recovery [11,17] as has been observed in the present investigation. However, pre-enrichment and enrichment had differential response in the recovery rate of the enteropathogens depending on sampling site and isolation media used. For example, when EMB was used as the recovery media, the efficiency of enrichment was better with the newly designed enrichment media compared to the conventional Hajna medium in case of sample taken from measuring point. However, in case of sample taken from oxidation lagoon 5, the recovery was slightly better with the conventional enrichment medium than with the newly designed enrichments. After enrichment with the newly designed media, the number of faecal coliform varied between 2.21×10^8 cfu/ml and 1.6×10^7 cfu/ml whereas with conventional enrichment, the number

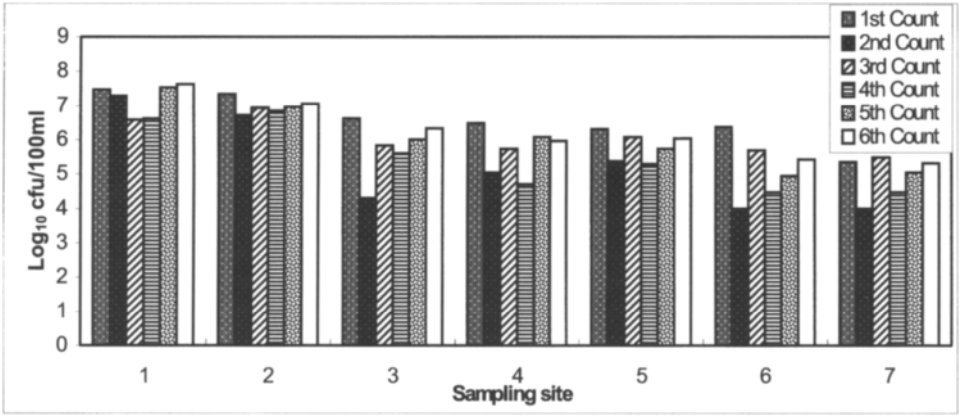

Figure 1. Efficiency of pre-enrichment and enrichment approaches in the recovery of enterobacteriaceae on XLD.

was lower and ranged between 1.3×10^6 and 2.1×10^5 cfu/ml. Similarly number of total heterotrophic bacteria, after pre-enrichment with PE-1, varied between 6.69×10^7 cfu/ml and 3.33×10^6 cfu/ml and the count was slightly better as compared to the conventional TH recovery approach.

The present investigation shows that the bacterial cell density was higher in the samples from the point where the treatment plant receives the sewage effluent, the number progressively decreased in the oxidation lagoons where sewage effluents are subjected to natural biological treatment by algae and other organisms but still remained beyond acceptable limits for discharge. In some cases the count at the discharge point in the river was higher. The cell count in the Buriganga river water was comparatively lower than that at the discharge points.

The average total coliform count of the seven sampling sites were 2.2×10^7 cfu/100ml, 1.0×10^7 cfu/100ml, 1.3×10^6 cfu/100ml, 9.6×10^5 cfu/100ml, 8.7×10^5 cfu/100ml, 5.3×10^5 cfu/100ml, 1.4×10^5 cfu/100ml respectively (Figure 2). The count of faecal coliform in sewage effluent even after treatment was high. The average faecal coliform count of the seven sampling sites was respectively 3.0×10^7 cfu/100ml, 9.2×10^6 cfu/100ml, 6.5×10^5 cfu/100ml, 1.1×10^6 cfu/100ml, 3.7×10^5 cfu/100ml, 8.4×10^4 cfu/100ml, and 4.4×10^4 cfu/100ml (Figure 3). Whereas the average total heterotrophic count on nutrient agar of the 7 sampling sites were respectively 2.7×10^8 cfu/100ml, 1.3×10^8 cfu/100ml, 1.5×10^7 cfu/100ml, $5.7 \times 10^{6\,cfu}$/100ml, 1.1×10^7 cfu/100ml, 2.8×10^7 cfu/100ml and 5.5×10^6 cfu/100ml (Figure 4).

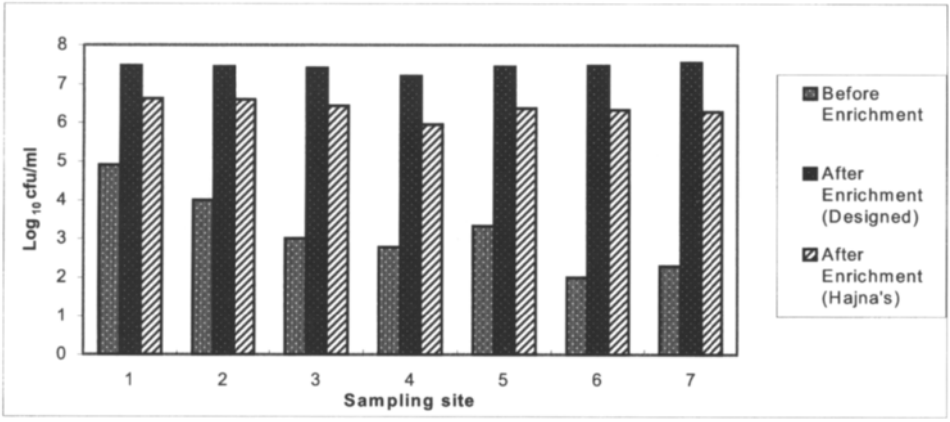

Figure 2. Total coliform counts from different sampling sites on MacConkey agar.

Table
Antibiotic sensitivity pattern of the isolated organisms

Name of the antibiotic (μg)	Total No. of organisms	No. of organisms sensitive	No. of organisms resistant	No. of organisms of intermediate sensitivity	% of organism sensitive	% of organism resistant	% of organism of intermediate sensitivity
Ampicillin (AMP, 10)	30	10	19	1	33.33	63.33	3.33
Amoxicillin (AMC, 30)	30	22	3	5	73.33	10	16.66
Bacitracin (BA, 10)	30	3	27	0	10	90	0
Ciprofloxacine (CIP, 5)	30	24	1	5	80	3.33	16.67
Doxycyclin (DO, 30)	30	10	10	10	33.33	33.33	33.33
Gentamycin (GM, 10)	30	29	1	0	96.67	3.33	0
Kanamycin (K, 30)	30	27	1	2	90	3.3	6.67
Nalidaxic acid (NA, 30)	30	11	7	12	36.67	23.33	40
Tetracycline (TE, 30)	30	18	6	6	60	20	20

The identified isolates belonged to the species of *Aeromonas, Bacillus, Citrobacter, Escherichia, Enterobacter, Klebsiella, Pasturella, Proteus* and *Pseudomonas.*

None of the *Escherichia coli* isolates was haemolytic or haemagglutination positive, which indicates that they were relatively non-virulent. Out of the five isolates none gave positive reaction to the polyvalent antisera of the different serogroups of *E. coli* examined.

From the results of the antibiotic sensitivity test, it was found that almost all the isolates were resistant to bacitracin. However, majority of them was susceptible to amoxicilin, ciprofloxacin, gentamycin and kanamycin. Some of them were susceptible while the others were resistant to ampicilin, doxycyclin, nalidixic acid and tetracycline (Table).

The density of faecal coliform both in sewage effluent and in the river water was high. According to WHO (1984) [18] standard, in drinking water, the number of faecal coliform should be zero per 100ml and in bathing water, the number should not exceed 200 per 100 ml. In the present investigation, counts of faecal coliform in the river water far exceeded the microbiological standard for both drinking and recreation purposes [19]. So it must be emphatically stated that the water from the river Buriganga was not only wholly unfit for drinking but also unfit for swimming, bathing or even washing of utensils.

The biological treatment site at Pagla reduces the number of total heterotrophs but that reduction was not significant in producing safe sewage effluent for discharging in the river Buriganga. In some cases the count at the discharge point was higher than that at the oxidation lagoons. This is in part due to the fact that the sewage treatment plant at Pagla has a by-pass from inlet to the discharge point. When the discharge flow from the treatment plant to the river Buriganga decreases, the by-pass is used to increase the flow rate by passing the sewage effluent from the inlet to the discharge point.

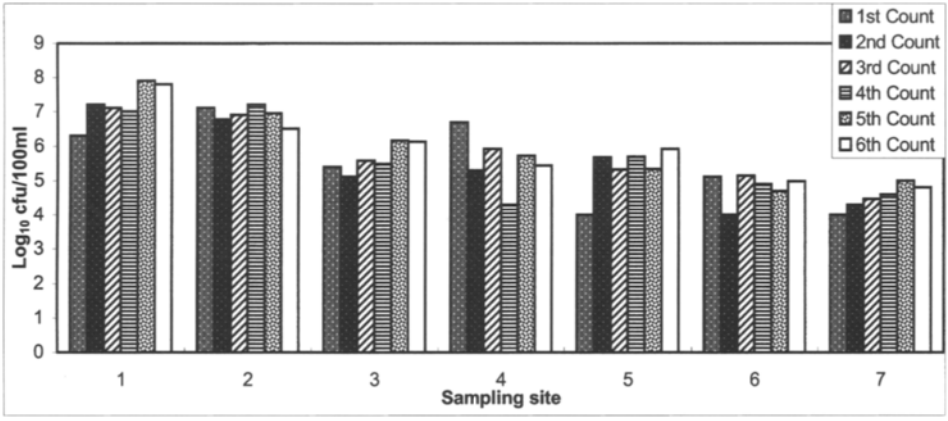

Figure 3. Faecal coliform counts from different sampling sites on mFC plates.

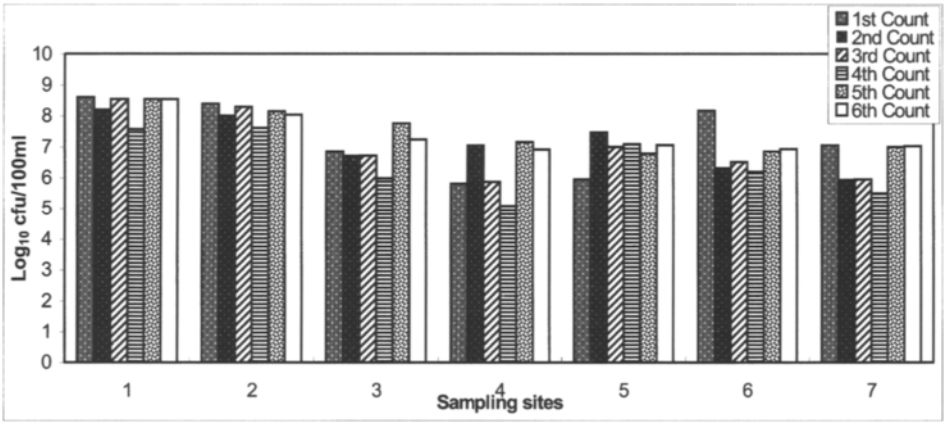

Figure 4. Total heterotrophic cell count from different sampling sites.

The newly designed pre-enrichment and enrichment approaches for increasing the recovery of stressed or injured enteropathogens and indicators were quite encouraging. However, repeated attempts to recover *Shigella* or *Salmonella* from sewage effluents or river water have not met with success. This may be due to the very fragile nature of *Shigella* that could not withstand the physio-chemical and biological stresses inherent in the sewage effluents [17]. In future attempts should be made for modification of these pre- enrichments using various growth stimulating and self-repair factors for the enrichment and isolation of *Shigella* and *Salmonella* from sewage effluents or river water.

The drug of choice for the treatment of diseases caused by such isolates will depend on their susceptibility to the drug. The modern high rate activated sludge process or intermediate waste management technology like mechanically aerated lagoons should be taken into consideration for the appropriate treatment facilities for domestic sewage near Dhaka.

REFERENCES

1. Ahmed, M. F., 1985. Waste disposal and degradation of water quality in and around Dhaka city. Proc. SAARC seminar, held in Dhaka. Ministry of Education, Govt. of People's Republic of Bangladesh, 176-182.
2. Luthfeara, 1995. Enrichment approaches to the isolation of *Salmonella*, *Shigella* and recovery of enteropathogens from treated sewage effluents. M.Sc. Thesis, Dept. of Microbiology, University of Dhaka.
3. Reazuddin, 1985. The state of environment in Bangladesh. Proc. SAARC seminar, held in Dhaka. Ministry of Education, Govt. of People's Republic of Bangladesh, 231-235.
4. UNDP - World Bank, UNDP and UN Children fund, 1993. Bangladesh situation analysis, Water Supply and Sanitation; World Bank Publication, Dhaka.
5. Asian Development Bank , 1990. Water supply and sanitation –beyond the decade. Asia and Pacific Regional Consultation ; 4-8 June, 1990. Published by ADB, 980 Manila, Philippines.
6. Henis,Y., Koteswara, R.G. and Alexander, M.,1989. Microb.Ecol.17:171-180.
7. Carrillo, M., Estrada, E. and Hazen, T.C.,1985. Microbiol. 50: 968-976.

8. Lopez-Terres,A.J., Hazen,T.C. and Toranzo, G.A. ,1987. Curr. Microbiol.15:312-316.
9. Flowers, R.S. and Ordal ,Z.J.,1979. J. Food Prot.42:362-367.
10. Ray, B., 1979. J. Food Prot.42: 346-55.
11. Busch,S.V. and Donnelly, C.W., 1992. Appl. Environ. Microbiol. 58: 14-20.
12. Brock, T. D., Madigan, M. T., Martinko, J. M. and Parker, J. (eds.), 1994. Biology of microorganisms. 7th edn., Prentice - Hall International, Inc., USA.
13. Hajna, A. A.,1955. Hlth. Lab., 13:83-89.
14. APHA, 1992. Standard methods for the examination of water and wastewater, 18th ed. American public health association, Inc. Washington D.C., USA.
15. Manual of Methods for General Bacteriology, 1981. 2nd printing, American Society for Microbiology, Washington D.C., USA.
16. Qadri, F., Haque, A., Faruque, S.M., Bettelheing, T.A., Brown, R.R. and Albert, M. J., 1993. J. Clinical Microbiol, 61:510-514.
17. Smith, J. L., 1987. J. Food Prot. 50:788-801.
18. World Health Organization, guidelines for drinking water quality, 1984. Volume 2: Health criteria and other supporting information, WHO, Geneva.
19. Bangladesh Standards and Testing Institution (BSTI),1993. Bangladesh standard specification for drinking water. BDS 1414, Dhaka.

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§3 Nutrient removal

Eutrophication problem is one of the most typical water pollution issues in many countries. Water environment in lake, coastal area and river is deteriorated by excess amount of nutrient that supports the excessive growth of phytoplankton. Water bloom or red tide is most explicit phenomenon of the eutrophication in which water ecosystem is disturbed.

Major causes of eutrophication are nitrogen and phosphorus. Discharging the wastewater containing these elements into water bodies can cause the eutrophication. Various processes including biological processes and physico-chemical processes have been proposed for removal of nitrogen or phosphorus from wastewater. Among these biological processes are promising because they utilize the function of microorganisms that exist in our natural environment.

Biological nitrogen removal process was proposed about 30 years ago but its history as full scale plant is shorter. Nowadays there are many full-scale plants of nitrogen removal working and its application is still increasing. Nitrogen removal process employs the functions of nitrifying organisms and denitrifying organisms. These organisms help each other properly in the process. Functions of each group of bacteria in the process are known to some extent. Although practical operation of the process shows no particular difficulties, further knowledge from the viewpoint of population dynamics of these bacteria is needed to improve the process.

New issue since late 80's is the emission of nitrous oxide which causes the global warming. We have to consider the other environmental aspect when we introduce wastewater treatment process. This issue teaches us the importance of overall or holistic evaluation of technology.

Biological phosphorus removal process employs unique function of complex microbial community. The bacteria accumulate and release phosphorus for their survival in stressed condition. The wastewater treatment technology should maximize this function by controlling the environmental condition for the bacteria. Biological phosphorus removal process is challenging from the viewpoint of research on microbiology. Microbial pathway and responsible bacterial species have been unknown. However, recent researches have revealed these basic mechanisms of this process. Understanding of bacterial physiology is essential in this process. This is the most typical example of contribution of microbiological knowledge to the process design and operation. Without the detailed knowledge on phosphate accumulating organisms, rational process design is impossible. Feasibility of biological phosphorus removal process largely depends on knowledge on complex bacterial community which possesses the function of phosphorus removal. Technological method to maintain such microbial community is practically essential.

The recent progresses in these nutrient removal processes are shown in this chapter. These processes are basically environmentally friendly, but we need proper design, operation and maintenance.

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Visualization of microscale distribution of nitrifying bacteria in biofilms formed in various type wastewater treatment processes

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Microbial ecology of nitrifying bacteria in various types of wastewater treatment processes and the dynamics of microbial ecology in biofilms were investigated by molecular biological techniques such as fluorescence *in situ* hybridization (FISH), visualization of viable bacteria using carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) and PCR based on method using magnetic beads. Nitrifying bacteria were found to exhibit various organizational forms under different conditions of substrate composition and concentration. Dynamics of microbial ecology in the biofilm caused by the gradual change of substrate composition was monitored remarking to the spatial distribution of ammonia-oxidizing bacteria and heterotrophic bacteria. Then visualization of viable bacteria in the biofilm stained by CFDA-SE combined with FISH was successfully demonstrated. Consequently, these techniques were applied to establish new method of fast tailoring highly effective nitrifying biofilm that is often quite difficult to be obtained on fluidized bed. Furthermore, PCR detection of ammonia-oxidizing bacteria using magnetic beads with selective purification of *amoA* was also successfully demonstrated. This novel technique enabled an elimination of interferences of PCR such as inhibitors of an amplification and coexisting DNA.

1. INTRODUCTION

Biological nitrogen removal from wastewater involves nitrification of ammonia to nitrite and nitrate followed by denitrification of them to nitrogen gas and release into the air. Although nitrification is a key process in wastewater treatment, retaining large amounts of nitrifying bacteria in the reactor for stable nitrification has not been established. This is caused by an extremely lower growth rate of lithoautotrophic nitrifying bacteria and their sensitivity to toxic shock, pH and temperature fluctuation, or unknown factor peculiar to a complex microbial community [1]. Generally, in order to improve nitrification ability, it is effective to form biofilm onto the supporting materials to keep large amounts of nitrifying bacteria in the reactor [2,3]. Furthermore, it is essential to elucidate the ecology and population dynamics of nitrifying bacteria in biofilms or activated sludge where various kinds of microbes form a complex microbial community.

The conventional method to detect the activity of biological reaction in the wastewater treatment process has relied heavily on the specific consumption rate of substrates. However, little attention has been directed to the number or spatial distribution of specific bacteria in

microbial community in the reactor. This is partly because difficulty of the characterization of bacterial populations in engineered system such as biofilms and activated sludge. The traditional culture-dependent monitoring techniques do not allow exact determination of the number and localization of specific bacterial cells because of bias of cultivation. In other words, a reactor or a treatment system has been so far regarded as a "black-box" .

The recent development of molecular biological techniques enabled scientists to overcome the limitation of the conventional methods [4-6]. Fluorescence *in situ* hybridization (FISH) is highly useful for detecting specific bacteria and analyzing complex microbial communities, due to the possibility of detecting specific bacterial cells by *in situ* hybridization with 16S rRNA-targeted oligonucleotide probes labeled with fluorescence. To date, FISH for detecting ammonia- and nitrite-oxidizing bacteria has been developed and successfully applied for their phylogenetical identification and quantification in environmental and engineered systems [7-10]. PCR technique has been used to detect bacteria in a variety of environmental samples at very low concentrations previously considered undetectable. This technique has been applied for detecting ammonia-oxidizing bacteria targeting 16S rRNA sequence and *amoA* (the gene encoding ammonia monooxygenase). However, complex microbial communities such as soil, activated sludge and biofilm contain coexisting DNA from other bacteria and inhibitors of PCR amplification that interfere precise amplification of target DNA. General extraction and purification protocol of DNA for PCR would cause problems, e.g., large fraction of DNA is lost during purification steps and large amount of contaminant DNA from other bacteria remains in purified DNA solution. To overcome these problems, a magnetic capture-hybridization PCR technique was reported to eliminate the inhibitory effect of contaminants in soil [11].

In this study, we investigated systematically spatial distribution of lithoautotrophic ammonia-oxidizing bacteria and other heterotrophic bacteria by FISH in various kinds of biofilms and activated sludge under different conditions of wastewater treatment processes. We analyzed samples from three types of reactor for nitrification which is biofilms on fluidized carrier beads in organic wastewater and in inorganic wastewater without nutrients and activated sludge in inorganic wastewater containing nutrients with lower ammonia loading rate. The dynamic response of ammonia-oxidizing bacteria and heterotrophic bacteria in the biofilm upon gradual change of substrate composition was also observed by FISH analysis. Furthermore, viable bacteria in the biofilm were visualized using CFDA-SE which is converted to a fluorescent product by intracellular esterase activity. Next, PCR detection of ammonia-oxidizing bacteria from complex microbial community was performed by targeting *amoA* sequence of DNA using magnetic beads. This method combines an initial DNA extraction and a purification step, using *amoA* DNA probes on magnetic beads and the subsequent PCR amplification step of the target gene.

2. MATERIALS AND METHODS

2.1. Reactor system

A complete mixing type three-phase fluidized bed bioreactor was used for the continuous nitrification processes [12]. A cement ball (diameter: 0.2 mm) or beads-shaped-carbon (BAC) was used as a carrier particle. The pH was maintained at around 7 by adding $NaHCO_3$. The temperature was maintained at room temperature to 30 °C.

Table 1
Composition of substrate and reactor conditions

		Reactor A	Reactor B	Reactor C
NH_4^+-N	(g/m^3)	500	600	250
TOC	(g/m^3)	0	0	1000
T-N	(g/m^3)	500	600	450
BOD	(g/m^3)	-	-	1500
Reactor volume	(*l*)	2	2	3
Hydraulic retention time (hr)		24	48	5
NH_4^+-N loading rate ($kg\ NH_4^+\text{-}N\ \cdot m^{-3}\ \cdot d^{-1}$)		0.5	0.3	1.2
Particles		Cement ball	BAC	Cement ball

Table 2
Time course of TOC /NH_4^+-N ratio of inlet solution and reactor condition.

Time (day)	TOC (g/m^3)	NH_4^+-N (g/m^3)	TOC /NH_4^+-N ratio (-)
- 0	1000	250	4.0
0 - 6	1000	500	2.0
6 - 12	750	500	1.5
12 - 18	500	500	1.0
18 - 24	250	500	0.5
24 -	0	500	0.0

Reactor volume: 2 *l*
Hydraulic retention time (HRT): 1 day

2.2. Feeding substrates

To compare the microbial ecology of nitrifying bacteria under different conditions, the following three types of mixed substrates were fed. Reactor A, inorganic wastewater <1> containing neither organic compound nor other nutrients such as phosphate; Reactor B, inorganic wastewater <2> containing nutrients without organic compound; Reactor C, organic wastewater containing ammonia as well as organic compound and nutrients. The operational condition and the substrate composition fed to each reactor are shown in Table 1. To examine the dynamics of microbial ecology formed in the biofilm, the thick biofilms containing both heterotrophic bacteria and nitrifying bacteria was used as a starting biofilm which had been acclimated in the organic wastewater of Reactor C for 6 month. Then, as shown in Table 2, total organic carbon (TOC) in the inlet solution was gradually reduced and finally led to zero.

2.3. FISH experiment

Biofilm and activated sludge samples for FISH analysis were immediately fixed in freshly prepared 4% paraformaldehyde solution for 20 hours. A biofilm section with 20 μm thickness was prepared using cryostat (Leica, CM 3050) at -20°C. Each slice was placed in each hybridization wells on a gelatin-coated microscopic slide and immobilized by air drying and dehydrating with 50, 80, 96% ethanol. (The activated sludge sample did not require this sectioning preparation).

The following 16S rRNA targeted oligonucleotide probes were used for *in situ* detection of ammonia-oxidizing bacteria and other bacteria, NEU23a [10] labeled with fluorescein

isothiocyanate (FITC): a probe specific for a region of the 16S rRNA of ammonia oxidizing bacteria targeting *Nitrosomonas europaea* etc.; NSO190 [7] labeled with X-rhodamine isothiocyanate (XRITC): specific for the region of the 16S rRNA of ammonia-oxidizing bacteria and EUB338 [13] labeled with Cy5: a probe for targeting all bacteria.

Hybridization of the environmental samples was performed according to the standard hybridization protocol described by Amann [14]. Finally, the microbial community analysis was carried out using a confocal scanning laser microscope (Leica, TCS NT) equipped with an Ar-Kr ion laser (488 nm, 568 nm and 647 nm).

2.4. CFDA-SE staining of biofilms

Immediately after sampling biofilms from the reactor, a biofilm section with 20 μm thickness was prepared as described above and placed on a gelatin coated microscopic slide. Each well on the slide where the sample was immobilized was filled with 5 μM of CFDA-SE solution for 30 min following rinsing with PBS to remove excessive CFDA-SE. To perform simultaneous staining with FISH, CFDA-SE stained sample was fixed in 4 % paraformaldehyde solution for 2 or 3 hours following hybridization steps described above.

2.5. PCR detection using Magnetic Beads

The two sets of primers for the amplification of probes and for the final detection of *amoA* gene were designed. The forward primer for the amplification of probes was labeled at 5' with biotin. DNA extracted from biofilms or sludge was set in PCR apparatus as a template for the amplification of 650-bp probe DNA with primer set labeled with biotin. Then amplified DNA probes labeled with biotin were bound on magnetic beads coated with streptavidin (Dynal A.S, Dynabeads). For the *amoA* gene detection from environmental samples, DNA was extracted from biofilms or sludge by boiling in 2.5% SDS solution. Then extracted DNA was hybridized specifically with DNA probes on magnetic beads. Therefore, the gene encoding *amoA* was purified selectively by washing magnetic beads on the magnet. Purified *amoA* gene was amplified by PCR with the primer set for detection of 570-bp amplification product. Finally, PCR products were electrophoresed and visualized in agarose gels.

3. RESULT AND DISCUSSION

3.1. FISH analysis of microbial ecology in each reactor

Figure 1 shows the FISH image of a biofilm in Reactor A; inorganic wastewater <1>. The thickness of the biofilm immobilized on particles was approximately 50 μm. A thin and soft biofilm made it difficult to obtain a precise cross section of the biofilm. Ammonia-oxidizing bacteria detected by the probes NEU23a and EUB338 (the white part) were observed as dominant population in the entire part of the biofilm. In contrast, other bacteria detected only by the probe EUB338 (the gray part) found to be significantly fewer than ammonia-oxidizing bacteria, probably because the lack of nutrients and organic compounds limited the growth of these bacteria. Figure 1(b) shows the magnified image of the microbial community in the biofilm. Each bacterium detected specifically by *in situ* hybridization could be confirmed as individual cells. Previously, it was reported that most species of ammonia-oxidizing bacteria in the environment samples tended to form mono-species clusters [7, 9]. However, the biofilms in Reactor A had two types of ammonia-oxidizing bacteria. One type of ammonia-oxidizers formed mono-species clusters and the other type existed individually. The probe NSO190 was also applied in an attempt to detect all species of ammonia-oxidizing bacteria.

As a result, ammonia-oxidizing bacteria stained by NSO190 were also stained by the probe NEU23a (data not shown) which indicated only *Nitrosomonas* sp. existed in reactor A as dominant ammonia-oxidizing bacteria.

Figure 2 shows the FISH image of the activated sludge sample in Reactor B; inorganic wastewater <2>. The white part shows *Nitrosomonas europaea / eutropha, or Nitrosococcus mobilis;* halophilic and halotolerant members of the genus *Nitrosomonas sp.* detected by both NEU23a and NSO190, while the gray part shows other ammonia-oxidizing bacteria detected by only NSO190 such as *Nitrosospira* sp.. The ammonia-oxidizers formed mono-species clusters. Various kinds of ammonia-oxidizing bacteria were observed from only Reactor B in this study, probably because the lower ammonia loading rate of Reactor B (0.3 kg NH_4^+-N · m^{-3} · d^{-1}) compared with other two reactors allowed the existence of two types of ammonia-oxidizing bacteria. One type of ammonia-oxidizing bacteria (*Nitrosomonas*) exhibited higher activity in higher concentration of ammonia and the other type (*Nitrosospira*) exhibited higher activity in lower concentration of ammonia [15]. Although reasons and advantages of various kinds of ammonia-oxidizing bacteria existing in complex microbial community are not sufficiently clarified, this result suggests the possibility of controlling the dominant species of ammonia-oxidizing bacteria in the reactor by ammonia loading rate.

Figure 3 shows the FISH image of the biofilm in Reactor C; organic wastewater. Most of the bacteria detected by only EUB338 (the gray part) could be considered as heterotrophic bacteria which occupied the outer part of the biofilm with about 100 μm thickness while ammonia-oxidizing bacteria detectable by both the probes EUB338 and NEU23a were distributed in the inner part of the biofilm. Ammonia-oxidizing bacteria and heterotrophic bacteria tended to be localized at different positions in the biofilms when the substrate contained a high concentration of ammonia as well as organic compound. Since heterotrophic bacteria has higher growth rate than autotrophic nitrifying bacteria, at first, heterotrophs occupied outer part of the biofilm where concentrations of dissolved oxygen and substrate are high enough for rapid growth of heterotrophs. In contrast, ammonia-oxidizing bacteria could exist only at the inner part of the biofilm due to their lower growth rate. It should be noted that even though the inner part of the biofilm might offer unfavorable conditions in terms of dissolved oxygen concentration, ammonia-oxidizing bacteria distributed moderately with high density. Figure 3b shows the magnified image of the microbial community in the biofilm. Each bacterium could be observed as individual cells. Two types of ammonia-oxidizing bacteria were also confirmed. One type of the ammonia-oxidizers formed mono-species clusters and the other type existed individually. Ammonia-oxidizing bacteria stained by NSO190 were also stained by the probe NEU23a (data not shown) which indicated only *Nitrosomonas* sp. existed in reactor C as dominant ammonia-oxidizers as well as microbial community in the biofilm of Reactor A.

3.2. Dynamic response of microbial ecology in the biofilm

The time course of NH_4^+-N and NO_x^--N concentrations and the thickness of the biofilm responses caused by the reduction of the TOC/NH_4^+-N ratio were shown in Figure 4 and 5.respectively. After the TOC/NH_4^+-N ratio reached 0, nitrification activity further improved, and concentration of NO_x^--N gradually increased up to 300-400 g/m^3. At day 50, nitrification rate was as high as 0.4 kg NH_4^+-N · m^{-3} · d^{-1} (Figure 4). Although the thickness of biofilm reduced from 300 μm to 200 μm at first, it remained stable after TOC/NH_4^+-N ratio reached 0 (Figure 5).

Figure 6 shows transition images of the microbial ecology in the biofilms caused by a

gradual reduction of the TOC/NH_4^+-N ratio. Figure 6a shows a FISH image of the starting biofilm acclimated in the substrate whose TOC/NH_4^+-N ratio was 4, containing a high concentration of ammonia and organic compounds. Heterotrophic bacteria detected only by EUB338 (the gray part) occupied the outer part of the biofilm with 100 μm thickness while ammonia-oxidizing bacteria detected by both EUB338 and NEU23a were distributed in the inner part of the biofilm, as described above. This microbial ecology slowly changed through a gradual reduction of the TOC/NH_4^+-N ratio. Figure. 6b shows a FISH image of the biofilm at a TOC/NH_4^+-N ratio of 1.5. Ammonia-oxidizing bacteria extended their existence area to the outer part of the biofilm previously occupied by heterotrophic bacteria. It appears that the decrease of organic compound limited the growth of heterotrophic bacteria, and consequently, ammonia-oxidizing bacteria could exist in the outer part of the biofilm.

When the TOC/NH_4^+-N ratio reached 0 at day 30, ammonia-oxidizing bacteria (the white part) maintained a large population in the biofilm and occupied the outer and inner part of the biofilm while heterotrophs still existed in the biofilm (Figure 6c).

After the TOC/NH_4^+-N ratio reached 0, transition of the microbial ecology still continued. At day 78 (48 days after TOC/ NH_4^+-N ratio reached 0), heterotrophic bacteria detected only by EUB338 were hardly observed in the biofilm in (Figure 6d). It was also found that, ammonia-oxidizing bacteria tended to exist in the outer part and at the bottom (contacted with a particle), and much fewer bacteria existed in the middle part. At day 78, nitrification activity was still kept as high as 0.4 kg NH_4^+-N $\cdot$ m^{-3} $\cdot$ d^{-1}.

As a result, the thick and highly active biofilm composed of high density of nitrifying bacteria could be obtained by gradual reduction of the TOC/NH_4^+-N ratio (Figure 5). This new procedure shorten the required time for tailoring the effective nitrifying biofilm to less than one-third time of the general biofilm formation carried out in Reactor A.

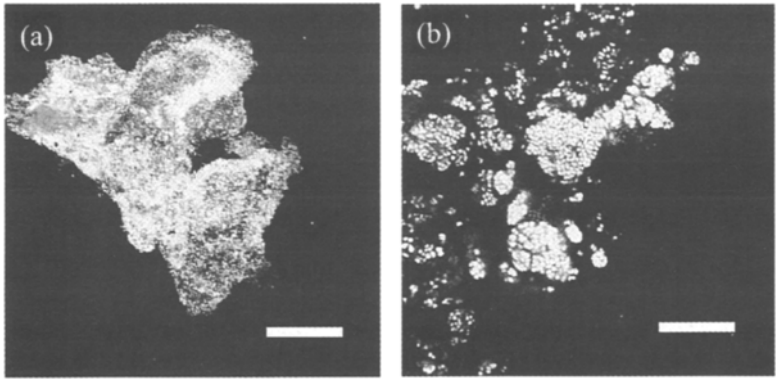


Figure 1. FISH image of biofilm in Reactor A, inorganic wastewater <1>. (a) FISH image of the entire biofilm, bar = 200 μm and (b) magnified image bar = 20 μm. The white part shows ammonia- oxidizing bacteria detected by both FITC-labeled probe NEU23a and Cy5-labeled probe EUB338. The gray part shows other bacteria detected only by the probe EUB338.

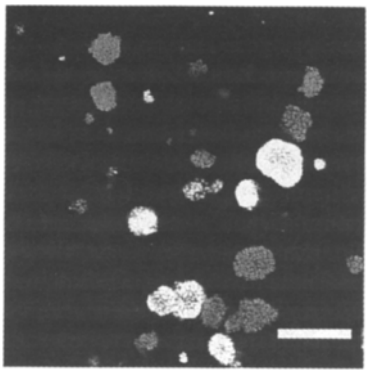


Figure 2. FISH image of activated sludge in Reactor B, inorganic wastewater. The white part shows ammonia-oxidizing bacteria detected by both FITC-labeled probe NEU23a and XRITC-labeled probe NSO190 (*Nitrosomonas europaea* etc.). The gray part shows other ammonia-oxidizing bacteria detected only by the probe NSO190, Bar = 20 μm.

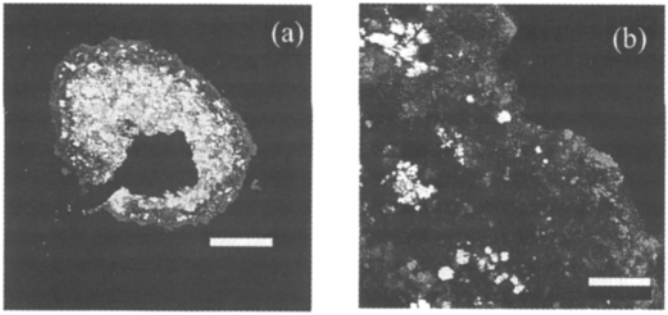


Figure 3. FISH image of the biofilm in Reactor C, organic wastewater. (a) FISH image of a cross section of the entire biofilm, bar = 200 μm and (b) magnified image showing each cell individually, bar = 20 μm. White part shows ammonia- oxidizing bacteria detected by both FITC-labeled probe NEU23a and Cy5-labeled probe EUB338. The gray part shows other bacteria detected only by the probe EUB338 (heterotrophs).

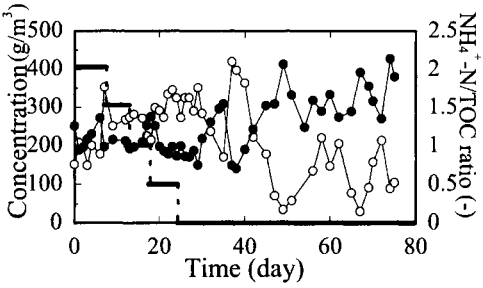


Figure 4. Time course of NH_4^+-N and NO_X^--N concentrations upon the reduction of the TOC/NH_4^+-N ratio. (o) Concentration of NH_4^+-N, (•) NO_x^--N, and (line) TOC/NH_4^+-N ratio.

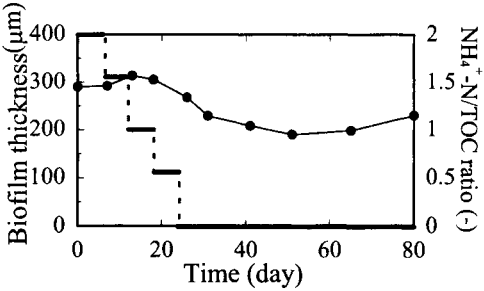


Figure. 5. Time course of biofilm thickness response upon the reduction of TOC/NH_4^+-N ratio. (•) Biofilm thickness and (gray line) TOC/NH_4^+-N ratio.

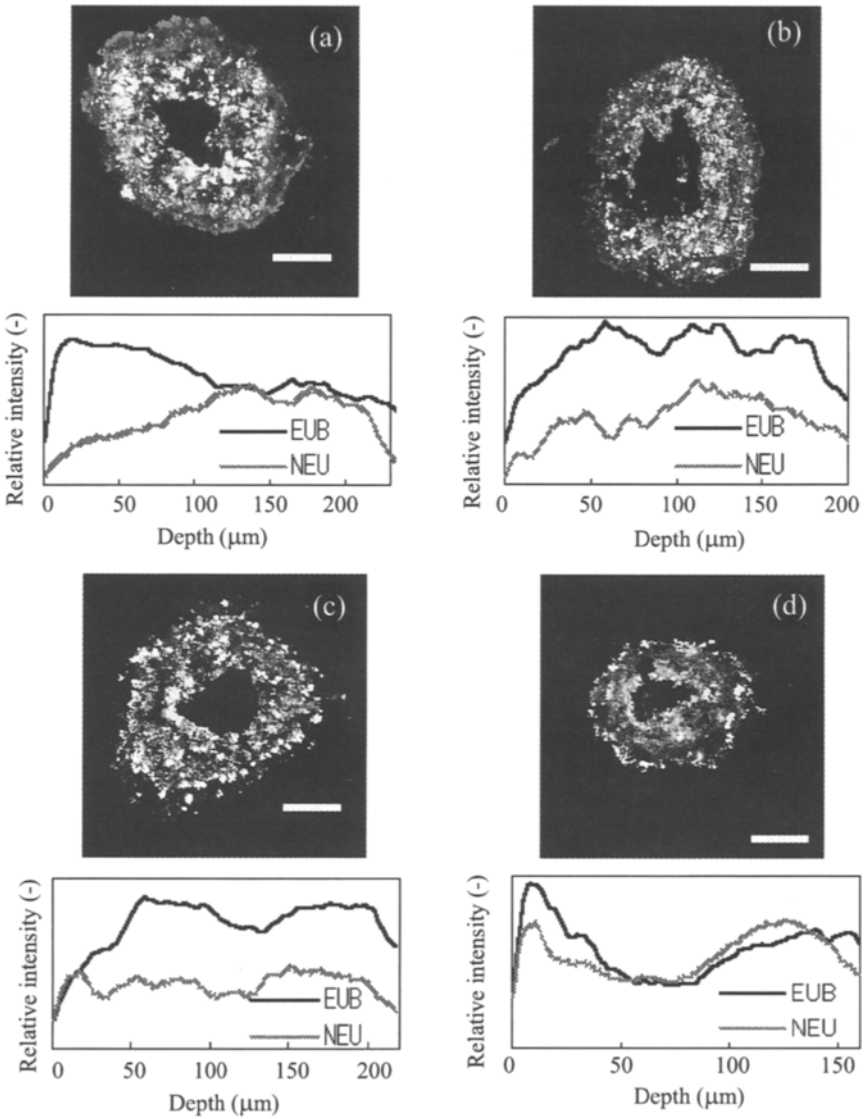


Figure. 6. Transition of the microbial ecology upon the gradual reduction of TOC/NH_4^+-N ratio observed by FISH analysis. (a) FISH image of the biofilm at TOC/NH_4^+-N ratio of 4, (b) 1.5, (c) 0 at day 30, (d) 0 at day 78. The white part shows ammonia-oxidizing bacteria detected by both FITC labeled probe NEU23a and Cy5-labeled probe EUB338. The gray part shows other bacteria detected by the probe EUB338 (heterotrophic bacteria). Bars represent 200 μm. The graphs under each image indicate relative intensity of fluorescence that is average of the fluorescence at a ringed band at each radial direction depth from the outer surface of the biofilm.

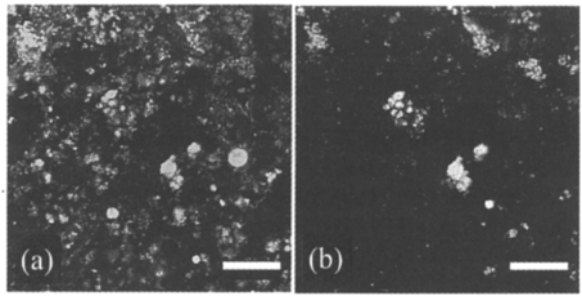
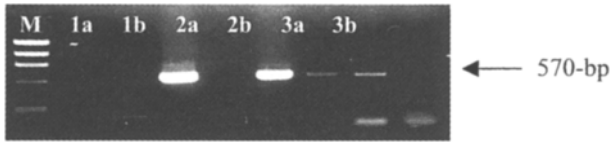


Figure 7. Image of the biofilm simultaneously stained by FISH and CFDA-SE. Bar = 20 μm. (a) image of bacteria in the biofilm stained by CFDA-SE, (b) FISH image of bacteria stained by the Cy5 labeled probe EUB338 after *in situ* hybridization. Both images are the same field of vision.



NO.	Samples
1a	Negative control <1> (without sample)
1b	Negative control <2> (without probes)
2a	Biofilms in Reactor A; inorganic wastewater with Magnetic beads
2b	Biofilms in Reactor A; extraction with phenol chloroform
3a	Biofilms in Reactor C; organic wastewater with magnetic beads
3b	Biofilms in Reactor C; extraction with phenol chloroform
4	*Nitrosomonas europaea*
5	*Pseudomonas putida*

Figure 8. Amplification of *amoA* gene and detection of ammonia-oxidizing bacteria by PCR using magnetic beads. Lane M is the marker ϕX174 *Hae*III.

3.3. CFDA-SE staining of biofilms

Viable bacteria in the nitrifying biofilm were visualized by the staining of CFDA-SE. The simultaneous staining of biofilm with FISH and CFDA-SE was successfully demonstrated with the successive steps (Figure 7). The bacteria stained by CFDA-SE were not always identical with the bacteria stained by the probe EUB338.

3.4. PCR detection with magnetic beads

Specific amplification of *amoA* from the biofilm sample in the reactors and pure-cultured sample of *Nitrosomonas europaea* was confirmed (Figure 8). PCR using magnetic beads technique was applied for detecting ammonia-oxidizing bacteria in the wastewater treatment reactor (reactor A and reactor C). In this method, it is possible to separate specific target DNA (encoding *amoA*) with high yield from other DNA and other interfering compounds. Consequently, total yields and sensitivity of the detection were greatly improved compared with PCR using general extraction and purification method of DNA (phenol chloroform method).

4. CONCLUSION

Microbial ecology and population dynamics of nitrifying bacteria in various kinds of wastewater treatment processes were analyzed using molecular biological techniques. As a result, the species and profile of ammonia-oxidizing bacteria were found to be strongly affected by the reactor operational conditions such as substrate composition and concentration. Dynamic response of the microbial ecology in the biofilm caused by the gradual change of substrate composition of feed was monitored remarking to spatial distribution of ammonia-oxidizing bacteria and heterotrophic bacteria. Then visualization of viable bacteria in the biofilm stained by CFDA-SE combined with FISH was successfully demonstrated. Consequently, it was elucidated that these techniques are powerful tools for evaluation and establishment of new method of fast tailoring of highly effective nitrifying biofilm. Furthermore, PCR detection of ammonia-oxidizing bacteria using magnetic beads with selective purification of *amoA* was also successfully demonstrated

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REFERENCES

1. T. Eighmy and P. L. Bishop, Water Res., 23 (1989) 947.
2. K. Noto, T. Ogasawara, Y. Suwa, and T. Sumino, Water Res., 32 (1998) 769.
3. S. Tsuneda, T. Miyoshi, Y. Aoi, and A. Hirata, Proceeding of IAWQ Asia-Pacific regional conference (1999).
4. G. A. Kowalchuk, Z. S. Naounenko, P. J. L. Derikx, A. Felske, J. R. Stephen and I. A. Arkhipchenko, Appl. Environ. Microbiol., 65 (1999) 396.
5. J. R. Stephen, G. A. Kowalchuk, M. V. Bruns, A. E. McCaig, C. J. Phillips, T. M. Embley, and J. I. Prosser, Appl. Environ. Microbiol., 64 (1998) 2958.
6. T. Takai, H. Niioka, Y. Gao, M. Matsumura, Y. Inamori, and A. Hirata, J. Jap. Soc. Wat. Environm, 20 (1997) 318.
7. B. Mobarry, M. Wagner, V. Urbain, B. Rittmann and D. A. Stahl, Appl. Environ. Microbiol., 62 (1996) 2156.
8. S. Okabe, H. Satoh and Y. Watanabe, Appl. Environ. Microbiol., 65 (1999) 3182.
9. A. Schramm, D. de Beer, M. Wagner and R. Amann, Appl. Environ. Microbiol., 64 (1998) 3480.
10. M. Wagner, G. Rath, R. I. Amann, H. -P. Koops and K. -H. Schleifer, Syst. Appl. Microbiol., 18 (1995) 251.
11. C. S. Jacobsen, Appl. Environ. Microbiol., 61 (1995) 3347.
12. A. Hirata and M. Noguchi, Water Sci. Technol., 30 (1995) 91.
13. R. I. Amann, L. Krumholz and D. A. Stahl, J. Bacteriol., 172 (1990) 762.

14. R. I. Amann, p. 1-15. A. D. C. In Akkerman, J. D. van Elsas and F. J. de Bruijin (eds.) Molecular Microbial Ecology Mannual. Kluwer Academic Publishers, Dordrecht (1995)
15. J. R. Stephan, A. E. McCaig, A. Smith, J. I. Prosser and T.M. Embley, Appl. Environ. Microbiol., 62 (1996) 4147

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Nitrous oxide production in nitrogen removal process treating domestic sewage from combined sewer system

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Nitrous oxide (N_2O) production was examined in bench-scale biological nitrogen removal process treating combined domestic sewage. Conversion from influent total nitrogen to N_2O was less than 0.1%. Shorter retention time and larger ratio of liquid recycle caused more emission. N_2O from nitrification step was generally greater than that from denitrification step in normal condition, but the latter sometimes showed abrupt high N_2O emission that lasted about a day. High ORP value was always observed in the occasion of such abrupt emission that occurred after inflow of rain water.

1. INTRODUCTION

Nitrous oxide (N_2O) is one of the effective greenhouse gases that cause the global warming. The atmospheric N_2O concentration in 1994 was 312 ppb and is increasing at a rate of 0.25%/yr [1]. Anthropogenic N_2O emissions have been significantly contributing to the increase of its atmospheric concentration [2]. Since N_2O has lifetime as long as 120 years [1] in the atmosphere and its radiative forcing is much greater than CO_2, its reduction is necessary.

Wastewater treatment processes are potential anthropogenic N_2O sources though the global emission rate from that source has not been precisely quantified. Hence, more data on the N_2O emissions from the source are needed. Moreover, the number of applications of nitrogen removal processes, by microbial nitrification and denitrification in most cases, to wastewater treatment processes is now on the increase. This may increase N_2O emissions from this source.

Total global N_2O emission is about 13-20 x 10^{12} g/year [3], namely 8-13 x 10^{12} g-N/year whereas nitrogen discharged as wastewater is approximately estimated to be 10 g/day x 365 days/year x $6x10^9$ (billion) = 22 x 10^{12} g-N/year. Therefore few percentage of conversion of wastewater origin nitrogen to N_2O -N can cause certain impact on global balance. IPCC tentatively shows the conversion percentage being 1% [4].

Nitrogen in wastewater causes eutrophication problem such as algal bloom or red tide in lakes or coastal area. Groundwater pollution by nitrate is in critical situation in many countries. There obviously exists need of removing nitrogen from wastewater. Among the various wastewater treatment technologies proposed, biological nitrogen removal process

using nitrification and denitrification is widely applied because of its effectiveness with reasonable cost and energy consumption. Nitrogen removal process with less emission of nitrous oxide production should be established.

2. PREVIOUS FINDINGS

2.1. Theoretical considerations

Biological nitrogen removal in wastewater treatment processes is carried out by microbial nitrification and denitrification, both of which can produce N_2O (Figure 1). In the course of nitrification, N_2O is produced as one of the by-products of NH_4^+ oxidation to NO_2^- [5]. Ritchie & Nicholas [6] showed that the principal mechanism of N_2O production by nitrifiers is NO_2^- reduction to N_2O by ammonium oxidizers, a process called 'nitrifier denitrification'. On the other hand, N_2O is produced as one of the obligatory intermediates in denitrification, and an N_2O/N_2 ratio produced by denitrification increases in condition that denitrification is suppressed because N_2O reductase is unstable compared to other reductases catalyzing denitrification [7].

In actual nitrogen removal from wastewater, proper combination of nitrification step and denitrification step is necessary. Nitrification and denitrification are very contrasting unit process in which type of bacteria and required condition are very different.

Availability of electron donor such as organic matters is essential condition for denitrification at high rate. Stoichiometric requirement of electron donor is 2.86 g oxygen equivalent/g-NO_3-N. However, value of about 3.5 is necessary for complete denitrification due to assimilation of organic matters to biomass. Practically COD/NO_3-N ratio in wastewater can be used to show this ratio. This ratio should be well higher than 3.5 in actual process because biodegradable fraction of COD is limited and because aerobic degradation of COD also takes place to some extent.

Nitrified liquid recycle type and intermittent aeration type are used as typical nitrogen removal processes for wastewater such as domestic sewage or night soil. Organic nitrogen and ammonia are nitrified and then denitrified using the organic matters contained in the wastewater.

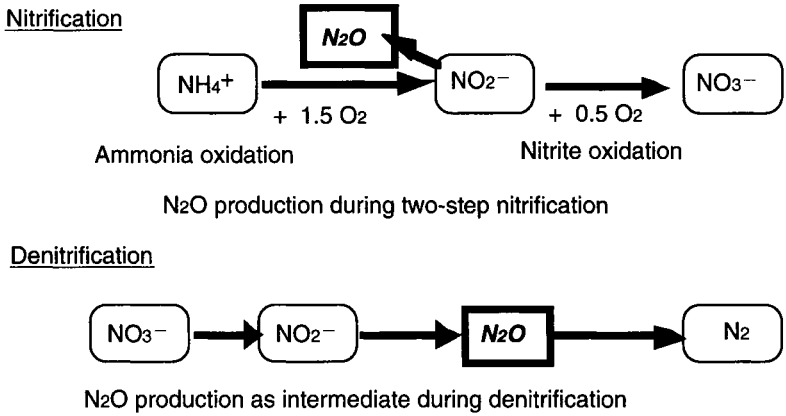


Figure 1. Nitrous oxide production in nitrogen removal.

2.2. Factors affecting N_2O production

Several laboratory experiments were carried out by the group of the authors [8-10] using synthetic wastewater to clarify the dependency of N_2O production on various conditions and parameters in nitrification and denitrification. As there are many full scale nitrogen removal processes in night soil treatment plants, very strong wastewater that contains about several hundreds milligram per liter of nitrogen and also high concentration of organic matters were used in these experiments.

Nitrification step

Potential factors affecting the nitrification are dissolved oxygen (DO), solid retention time (SRT), pH and presence of organic matters that are degraded by heterotrophic organisms competitively consuming the oxygen.

Low concentration of dissolved oxygen (DO) and short solid retention time (SRT) promoted N_2O production by nitrifying activated sludge (Figure 2) [9]. Conversion of ammonia to N_2O has reached as high as 8 percent at DO lower than 1 mg/l. Solid retention time showed more significant effect on N_2O production. Nitrite accumulation was observed when N_2O was produced. These experiments indicated that there is a risk of significant amount of N_2O production under stressed condition. Another series of experiment showed that presence of organic matter during the nitrification caused N_2O production [9]. Operation with low DO, short retention time and simultaneous organic oxidation are conditions for efficient nitrification process operation. This suggests that the prevention of N2O production becomes more important in efficient and innovative nitrogen removal process.

Denitrification step

Denitrification is another potential occasion of N_2O production. Low COD/NO_3-N ratio in the wastewater, short SRT, and low level of pH promoted N_2O production by denitrifying activated sludge (Figure 3) [8]. Among these parameters, COD/NO_3-N is especially important factor governing completeness of denitrification. It is reasonable that N_2O can be produced as intermediate when denitrification reaction stops midway due to insufficient organic matters. Conversion from nitrate to N2O was higher than 10% at short SRT and low pH value.

It was also reported by von Schulthess *et al.* [11] that higher DO resulted in more N_2O production by denitrifying activated sludge.

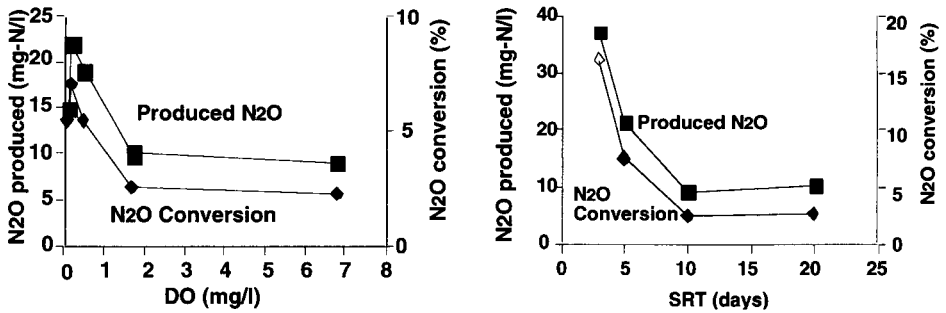


Figure 2. Effect of dissolved oxygen and solids retâention time on nitrous oxide production during nitrification of strong wastewater[9].

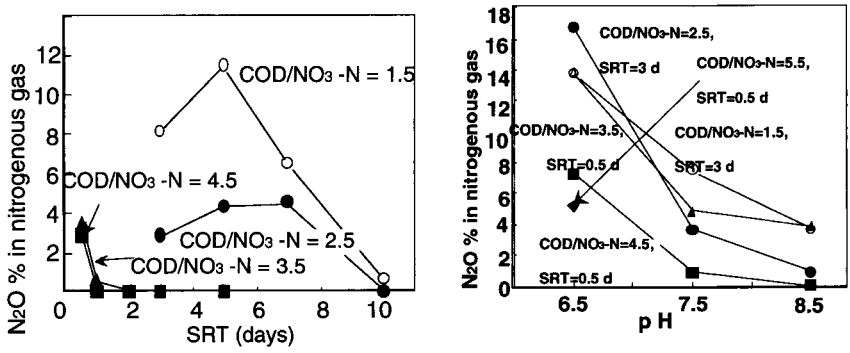


Figure 3. Effects of COD to nitrate ratio, solid retention time and pH on N_2O production during denitrification of strong wastewater [8].

Nitrification and denitrification combination

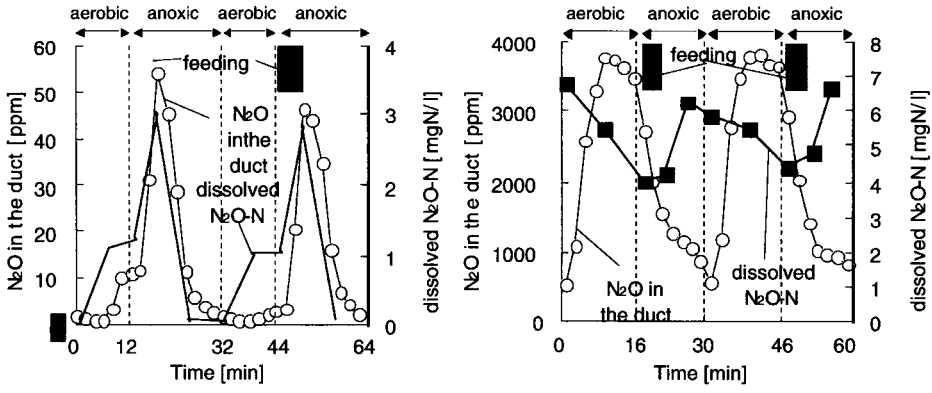
The above results were from activated sludge when either nitrification or denitrification occurred in separated condition. However, most of the nitrogen removal process employs both nitrification and denitrification in combination. Itokawa *et al.* [10] showed that 20-30% of ammonium nitrogen can be converted to N_2O in denitrification step of intermittent aeration process treating strong synthetic wastewater which has COD/NO_3-N ratio was 3.5. COD/NO_3-N ratio of 5 was satisfactory for complete denitrification in these experiments.

2.3. Survey of full-scale wastewater treatment plant

Laboratory experiment can not demonstrate the dynamic response of nitrous oxide production against perturbed input such as disturbance to the process, fluctuation in influent quality. However, actual treatment plant always has such perturbation. The experience during the laboratory experiment suggested that nitrous oxide is highly sensitive to change of condition.

The authors' group has shown that significant amount of N_2O can be emitted from night soil treatment plants, which are the conventional treatment site of human excreta in Japan [12]. The wastewater is characterized by high concentration of nitrogen (5,000-6,000 mgN/l of total nitrogen) and organic matter (3,000-5,000 mg/l of COD_{Mn}), and small C/N ratio of the influent. High-rate intermittent process was used in the treatment plant. The pattern and amount of nitrous oxide production during the intermittent aeration process varied (Figure 4) with occasion at same treatment facility. It was generally shown that nitrous oxide was produced when either denitrification or nitrification was incomplete.

Survey of N_2O emission from domestic sewage treatment is limited. It seems that the conversion percentage from influent nitrogen to N_2O is lower than the case of strong wastewater. The sewage treatment plant that employs the biological nitrogen removal process is potential source of N_2O, but the other potential source is conventional activated sludge plant where nitrification takes place without intention. The operation of the latter process does not take into account the proceeding of nitrification and denitrification, but these reactions actually take place in rather incomplete manner. Partial nitrification often occurs in the activated sludge reactor during the summer period due to the high activity of nitrifying bacteria, and denitrification can take place in settling tank once nitrification happens.



(a) Survey 3
(aerobic: 12 min; anoxic: 20 min,
duct flow = 390 m ^{3}/h)

(b) Survey 4
(aerobic: 16 min; anoxic: 14 min,
duct flow = 1040 m ^{3}/h)

Figure 4. N_2O concentrations in the duct of the intermittent aeration tank and dissolved in the mixed liquor [12].

Presence of nitrite in the effluent from sewage treatment plant is sometimes observed in summer period. Such "unintentional" nitrogen transformation has higher possibility of nitrous oxide emission than the intentional nitrogen removal process with proper control. This high possibility of nitrous oxide production can be expected from the research findings of high nitrous oxide production during incomplete nitrification or denitrification.

3. BENCH-SCALE STUDY USING DOMESTIC SEWAGE

Bench-scale experimental units were installed at an experimental station located in Shibaura Sewage Treatment Plant in Tokyo receiving combined sewage.

3.1. Materials and methods

Experimental unit

The circulation type of process known as Bardenpho process was used in this study. The process consists of anoxic reactor followed by aerobic reactor. The mixed liquor from aerobic reactor that contains nitrate was returned to anoxic reactor and mixed with fresh wastewater that contains fresh organic matters to ensure the denitrification process. As shown in Figure 5, settled and screened domestic sewage was continuously fed to the reactor system installed within a building. Hollow fiber membrane was used for solid and liquid separation. Gas was circulated through closed vessel to ensure complete mixing and equilibrium condition of N_2O between gas phase and dissolved form. Small amount of fresh nitrogen gas or air was continuously supplied to the closed reactor to maintain anoxic or aerobic condition of the reactor. Certain solids retention time (SRT) was maintained by withdrawing the mixed liquor from the aerobic reactor. Changed parameters were hydraulic retention time (HRT), SRT and liquid circulation ratio (CR) which is defined as the percentage of amount of recycled flow from the aerobic reactor against the wastewater inflow. The pH in the aerobic reactor was automatically controlled at no lower than 6.5. Although

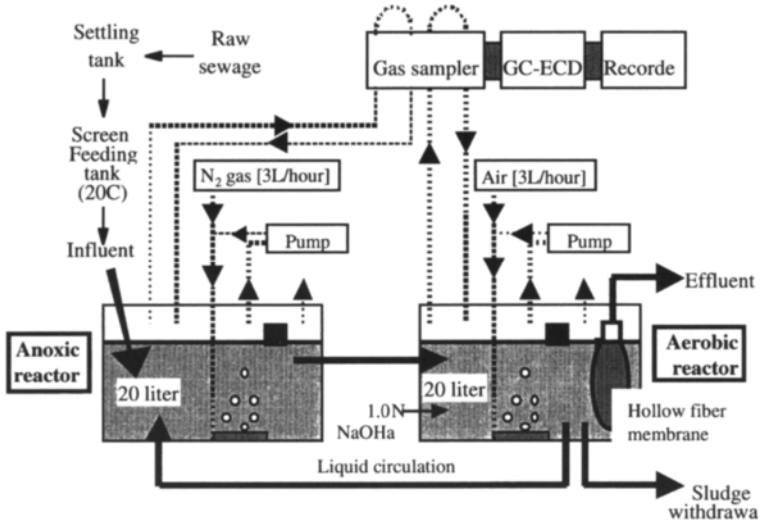


Figure 5. Experimental set up of circulated denitrification reactor.

the actual process usually has additional denitrifying reactor to improve nitrate removal, it was not included in the experimental unit to make the interpretation of the results simple.

The influent to the experimental reactors was domestic sewage from a combined municipal sewer system. Characteristics are shown in Table 1. The average COD/T-N ratio was about 5, that was theoretically satisfactory for nitrogen removal, but not high enough to always guarantee the situation of excessive availability of degradable organic matter from the viewpoint of denitrification.

As the experimental units received combined sewage, influent quality was influenced by rainfall or snowfall.

Process monitoring

Nitrous oxide was automatically monitored in the interval of two hours using GC-ECD without radioisotope. Redox potential (ORP) was also monitored continuously.

3.2. Results and discussion

Table 2 summarizes the nitrogen removal of the recycled reactor. As the secondary denitrifying reactor was not used in this experimental unit, nitrate remained in the effluent. Overall nitrogen removal was satisfactory in all examined conditions.

Continuous monitoring of N2O

Table 1.
Average characteristics of the influent

Total COD (mg/l)	Dissolved COD (mg/l)	Total N (mg/l)	NH4-N (mg/l)	NO2-N (mg/l)	NO3-N (mg/l)
152	55	31	17	0	0.1

N_2O in both aerobic and anoxic reactor was kept well low in most of the time. However, abrupt increase in N_2O production which lasted for about one day was observed in the denitrifying process. This also caused the N_2O increase in the aerobic reactor because it received the effluent from the anoxic reactor. Typical example of such high N_2O is shown in Figure 6. Aerobic reactor itself did not cause such sudden increase of N_2O. Continuous monitoring was very effective for the control of N_2O emission because such abrupt increase is hard to be observed by spot monitoring of N_2O. This abrupt high N_2O emission happened after the event of rainfall, although rainfall did not always cause such high N_2O production. Dilution of sewage by rainwater could be the main reason of N_2O production. Nitrification

Table 2.
Removal of nitrogen and organic matters

Run	B	A	C	E	D	F
HRT (h)	12	12	12	6	6	6
SRT (d)	20	20	20	10	10	10
Recycle ratio (%)	100	200	400	100	200	400
Effluent from anoxic reactor						
NH_3-N (mg/l)	7.8	8.4	8.8	7.0	7.6	9.6
NO_2-N (mg/l)	0.1	0.1	0.1	0.0	0.1	0.1
NO_3-N (mg/l)	0.1	0.1	0.3	0.1	0.1	0.4
Dissolved COD (mg/l)	15.5	16.2	16.9	17.8	18.9	19.2
Effluent from aerobic reactor						
NH_3-N (mg/l)	0.1	0.1	0.2	0.2	0.2	0.7
NO_2-N (mg/l)	0.1	0.4	0.3	0.1	0.1	0.3
NO_3-N (mg/l)	10.6	9.2	9.2	8.5	7.6	6.6
Dissolved COD (mg/l)	11.8	13.3	14.0	15.2	15.6	14.9

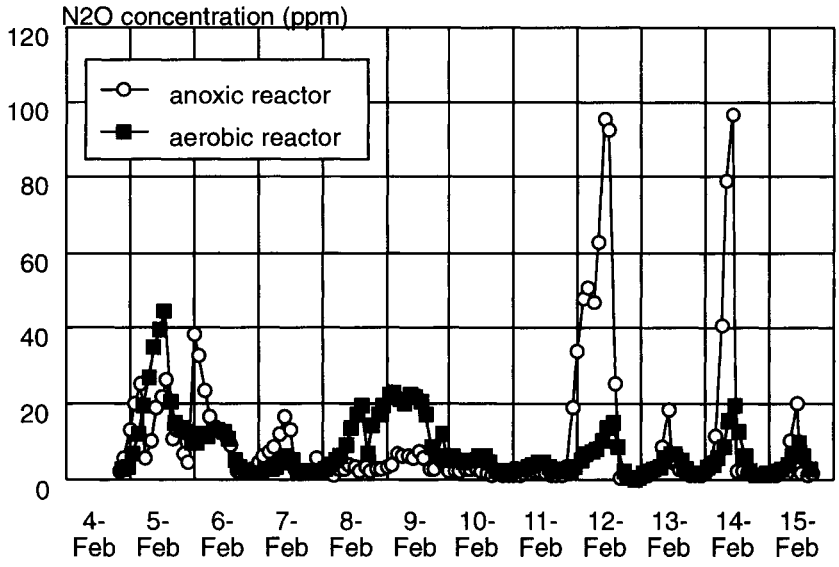


Figure 6. Nitrous oxide concentration in gas phase of anoxic and aerobic reactors (Run A).

did not show such sensitive response to the rainfall.

Table 3 summarizes the monitoring data during the steady state of each condition. Maximum values were far higher than the average value in all cases in anoxic reactor. This was because of the occurrence of sudden increase of N_2O mentioned before.

The observed N_2O concentration data (sampling interval: 2 hours) were categorized to concentration group with range of 2 ppm or 4 ppm, and their frequency distribution is summarized in Figures 7 and 8 . Although there were certain events of very high concentration, N_2O was kept most of the time very low in the anoxic reactor. The frequency distribution consisted of two parts. The first part was smooth distribution in very low concentration and the second part was certain frequency at the highest range of concentration. It seems reasonable to divide N_2O production in the anoxic reactor into normal period and high concentration events.

The N_2O concentration in aerobic reactor experienced no such extremely high value, but was higher than that in anoxic reactor in normal period. This indicates that N_2O was mainly produced in the aerobic reactor with nitrification. The frequency distribution in the aerobic reactor shifted to higher concentration when circulation ratio was increased. The distribution was rather flat in the aerobic reactor with SRT of 10 days. This suggests that shorter SRT brought about more N2O production in the nitrification step, although the concentration was not very high.

Amount of N_2O emission

Based on continuous monitoring of N_2O concentration in both reactors and the amount of gas release from the reactors, total amount of N_2O emission was estimated. The calculation was done for steady state period including the high N_2O emission events in the anoxic reactor. Emission from the aerobic reactor is divided into the case with N_2O concentration lower than 40 ppm (namely normal period) and that higher than 40 ppm (namely high emission event) and compared in Figure 9. The value can be compared directly because the N_2O emission is

Table 3.
Gaseous N_2O concentration in anoxic and aerobic reactors.

Run	B	A	C	E	D	F
HRT (h)	12	12	12	6	6	6
SRT (d)	20	20	20	10	10	10
Recycle ratio (%)	100	200	400	100	200	400
Gas phase in anoxic reactor						
Average (ppm)	0.80	4.87	2.91	2.03	10.70	15.90
Median (ppm)	0.59	2.00	0.69	1.94	2.85	4.84
Maximum (ppm)	30.47	96.76	96.73	12.40	142.3	153.8
Standard deviation (ppm)	1.62	11.00	10.92	1.06	20.92	30.71
Number of data	409	418	116	241	185	336
Gas phase in aerobic reactor						
Average (ppm)	1.63	4.81	5.98	16.25	19.44	28.31
Median (ppm)	1.15	2.52	5.53	14.55	15.79	9.79
Maximum (ppm)	6.92	44.43	26.97	75.16	64.80	286.2
Standard deviation (ppm)	1.23	5.93	4.27	9.60	13.35	56.79
Number of data	411	418	117	240	185	336

Monitoring data were obtained every two hours.

expressed as per volume of treated wastewater. It should be noted here that the emissions from the anoxic and aerobic reactor do not solely represent the reaction of denitrification and nitrification, respectively. As solubility of N_2O is very high, the liquid carried dissolved N_2O from one reactor into the other reactor, the N_2O produced can be released in the following reactor. Nevertheless, the N2O release from each reactor approximately provides information on N_2O production in each reaction.

Higher loading (HRT 6h, SRT 10 d) caused more N_2O production in general. The emission from aerobic reactor was more significant than that from anoxic reactor in normal

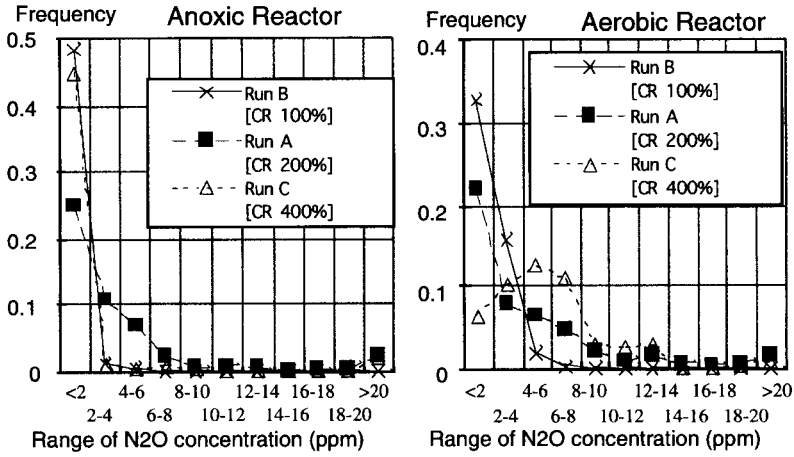


Figure 7. Frequency distribution of N_2O concentration in anoxic and aerobic reactors with various circulation ratio (CR). [HRT = 12h, SRT = 20d]

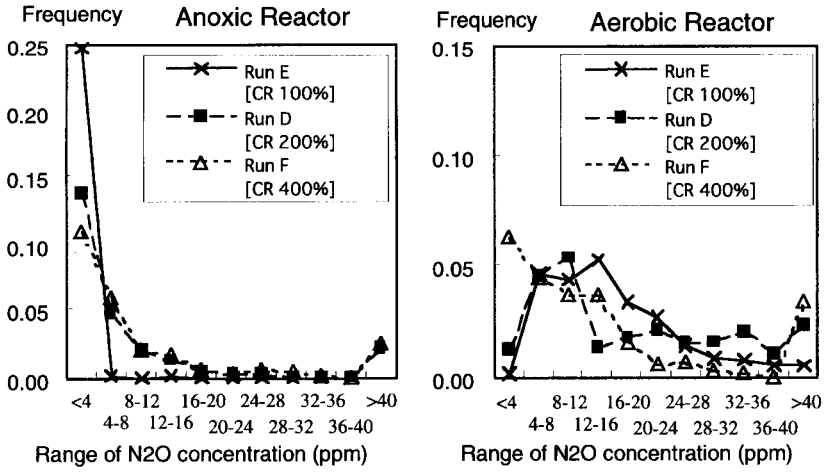


Figure 8. Frequency distribution of N_2O concentration in anoxic and aerobic reactors with various circulation ratio (CR). [HRT = 6h, SRT = 10d]

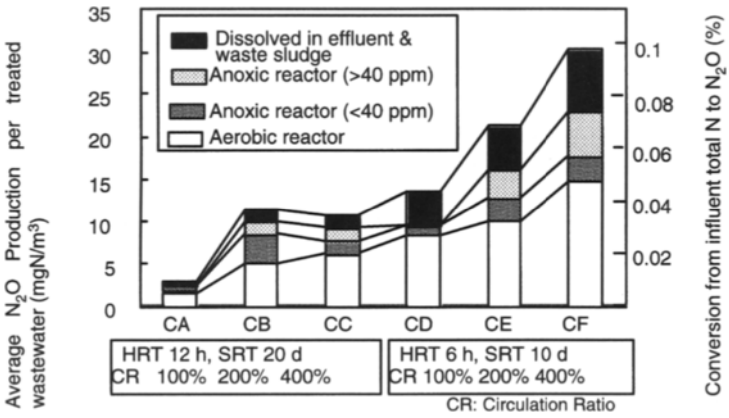


Figure 9. Contribution of aerobic reactor, anoxic reactor with low concentration, and anoxic reactor with high concentration to N2O procution.

condition. However, high emission events in anoxic reactor contributed to some extent especially when circulation ratio was high.

N2O emitted to the environment with the final effluent in the dissolved form is estimated based on theoretical solubility and measured partial pressure of N2O in the head space of the reactor. Taking this soluble N2O into account, the maximum N2O emission in this study was about 30 mgN/m3 at circulation ratio of 400% and HRT/SRT being 6 h/10 d. This amount accounts for about 0.1% of the influent total nitrogen. This percentage was lower than the tentative value (0.1%) shown by IPCC [4].

N2O emission and ORP

The above results indicate that high emission events during the denitrification should be avoided. If such event were monitored easily even without measuring N2O emission, it would help better control of N2O emission from the whole treatment system.

Figure 10 shows the correlation between monitored ORP and N2O concentration in the anoxic reactor. ORP should be normally well below -200 mV in denitrification process. Many of the data were below -100 mV, but ORP became higher than +200 mV in some cases. All the data of high N2O happened together with ORP higher than 300 mV although there were also many cases of normal N2O concentration in spite of high ORP. Because ORP value and denitrification reaction influence each other, it is not obvious whether high ORP caused the poor denitrification with N2O production or poor denitrification caused high ORP. ORP can be a useful indicator showing the possibility of high N2O emission in denitrification process.

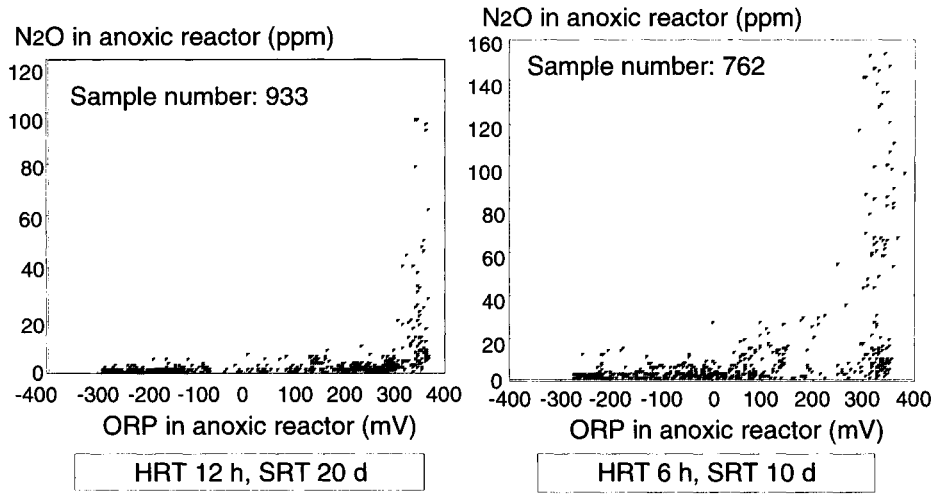


Figure 10. Correlation between ORP and N_2O concentration in anoxic reactor.

CONCLUSION

Nitrous oxide (N_2O) production from nitrogen removal process treating combined domestic sewage was examined. The followings are major findings.

Overall N_2O production was less than 0.1% of the inflow total nitrogen at SRT no shorter than 10 days. This conversion percentage is well acceptable.

N_2O emission from nitrification was normally greater than that from denitrification. However, denitrification step sometimes experienced events of very high N_2O emission lasting about the order of one day. There existed correlation between ORP and this high N_2O emission that happened at ORP higher than +300 mV.

N_2O emission increased with decrease of SRT and with higher ratio of liquid circulation. Maintaining satisfactory SRT and operating the process with reasonable circulation ratio reduce N_2O emission.

REFERENCES

1. IPCC (1996), "Climate Change 1995: The Science of Climate Change. Contribution of Working Group I to the Second Assessment, Report of the Intergovernmental Panel on Climate Change." Cambridge University Press
2. Khalil,M.A.K. and R.A.Rasmussen (1992). J. Geophys. Res., 97, 14651-14660.
3. Intergovernmental Panel on Climate Change (IPCC) (1995), "Climate Change 1994: Radiative Forcing of Climate Change and an Evaluation of The IPCC IS92 Emission Scenarios.", Cambridge University Press.
4. Intergovernmental Panel on Climate Change (IPCC) (1996) Revised 1996 IPCC Guidelines for National Greenhouse Gas Inventories - Reference Manual, Section 4.5.4.
5. Prosser,J.I. (1989). Adv. Microbiol. Physiol., 30, 125-181.

6. Ritchie,G.A.F. and D.J.D.Nicholas. (1972). Biochem. J., 126, 1181-1191.
7. Knowles,R. (1982). Denitrification. Microbiol. Rev., 46, 43-70.
8. Hanaki,K., Z.Hong and T.Matsuo. (1992). Wat. Sci. Tech., 26 (5/6), 1027-1036.
9. Zheng,H., K.Hanaki and T.Matsuo (1994). Wat. Sci. Tech., 30 (6), 133-141.
10. Hiroki Itokawa, Keisuke Hanaki and Tomonori Matsuo : Nitrous oxide production in high-loading biological nitrogen removal process under low COD/N ratio condition, Water Research, in printing.
11. von Schulthess,R., D.Wild and W.Gujer (1994). Wat. Sci. Tech., 30 (6), 123-132.
12. Itokawa,H., K.Hanaki & T.Matsuo (1996) Wat.Sci.Tech., 34 (1-2), 277-284.

Quinone profile analysis of activated sludge in enhanced biological P removal SBR treating actual sewage

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The quinone profile technique was applied to investigate the microbial community of activated sludge in anaerobic/aerobic SBR treating actual sewage. enhanced biological phosphorus removal (EBPR) sludge was developed with the addition of acetate to influent sewage and then the sludge was exposed to lower acetate addition condition. Deterioration of biological P removal was evaluated by monitoring effluent quality. Change in concentration of polyphosphate accumulating organism (PAO) was estimated by application of a kinetic equation in the IAWQ activated sludge model No.2 to the results of phosphorus release tests. The change of PAO content in sludge was compared with variation of quinone species during the disturbed period with the lower addition of acetate. Although fluctuation of two menaquinone species (MK-8(H4) and MK-7) looked relatively similar to change of PAO content, there was no clear relationship between them. The quinone profiles of the SBR sludge were also compared with those of activated sludge in other processes that were not designed for EBPR.

1. INTRODUCTION

It is well known that alternating anaerobic/aerobic conditions in sequencing batch reactor (SBR) activated sludge processes can help in achieving enhanced biological phosphorus removal (EBPR) [1]. In order to achieve stable EBPR, it is very important to evaluate the relationship between microbial population dynamics in activated sludge and process operation. Recently mathematical modeling and simulation became popular for effective control and operational optimization of the SBR for nutrient removal. Operational scenarios may be tested by simulation before conducting experiments on trial and error base.

The most successful activated sludge model, so called Activated Sludge Model No.1 (ASM1) [2], was developed in 1986 by the task group for mathematical modeling for design and operation of biological wastewater treatment of the IAWPRC (now IWA). This approach has been accepted by a wide range of engineers and applied successfully to the SBR system (eg. Oles and Wilderer, 1991[3]). The ASM1 has been updated to a higher version named Activated Sludge Model No.2 (ASM2), which includes phosphorus removal [4]. The latest version, Activated Sludge Model No.3 (ASM3), was proposed to express substrate storage phenomena [5].

We experimentally investigated the deterioration of EBPR caused by remaining nitrate in SBR process [6]. In this study, we succeeded in simulating long-term dynamic behavior of

nutrient removal using the SBR model based on ASM2. The simulated results implied that the deteriorating phenomena of phosphorus removal could be explained by two mechanisms; poor P-uptake by polyphosphate accumulating organism (PAO) and washout of PAO itself. Once a well calibrated SBR model is developed, simulation of different operating strategies for phosphorus removal, such as adjustment of anoxic/anaerobic phase under steady state and transient loading conditions can be easily performed rather than conducting lab-scale experiments. However, we could not achieve satisfactory level of model calibration, because each fraction of microbial group has still not been calibrated in the model simulation.

The quantitative determination of each bacterial concentration in activated sludge experimentally has been very difficult, while the activated sludge models incorporate several microbial groups such as heterotrophic organisms, nitrifiers, and PAOs. Identification and characterization of microorganisms in activated sludge by isolation techniques are considered to be very difficult. A chemotaxonomic approach using the quinone as biomarker has provided invaluable information on microbial population dynamics in activated sludge system [7]. We have investigated the structure and dynamics of microbial community in SBR activated sludge process for EBPR treating a synthetic wastewater containing acetate and pepton as main organic substrates.

We found out that ubiquinone with 9-isoprene unit (Q-9) and menaquinone with 7-isoprene (MK-7) were well related to the development of EBPR process after the introduction of anaerobic /aerobic operation. However, microbial communities in activated sludge seem to be very much dependent on substrate composition [8], [9]. Hiraishi *et al.* (1998)[8] reported that quinone profiles in activated sludge processes might be affected more by the nature of the influent wastewater than by the introduction of an anaerobic stage into the process. It is therefore necessary to investigate quinone profile of EBPR activated sludge treating actual sewage containing more colloidal and particulate organic matters and compare the results with activated sludge process which are not designed to incorporate anaerobic phase in their treatment facilities.

Although we found several possible indicators of quinone species for biological P removal activity, the quinone species do not directly correspond to predominant quinone of PAOs. However, it is useful to discuss the effectiveness of quinone profile analysis for evaluation of growth or decay of PAOs as well as behavior of EBPR activity. The objectives of this study on activated sludge in SBR treating actual sewage are listed as follows;

1) To investigate response in quinone profile of the activated sludge to reduction of added acetate as substrate.
2) To compare their quinone profiles with those of sludge in other processes.
3) To discuss the profiles change and relationship between estimated PAO content and quinone fraction to find possible markers for EBPR activity or PAO.

2. MATERIALS AND METHODS

2.1. Experimental apparatus and operational conditions

An anaerobic/aerobic SBR process was operated for biological phosphorus removal from actual sewage. The duration of the total cycle was 6 hours consisting of four phases such as anoxic/anaerobic mixing phase (2.0 hrs) including wastewater feed (1.0 hrs) at a constant rate, the aeration phase (3.0 hrs), settling phase (0.6 hrs) and discharge phase (0.4 hrs) as shown in Figure 1. The activated sludge for inoculation was collected from a conventional activated

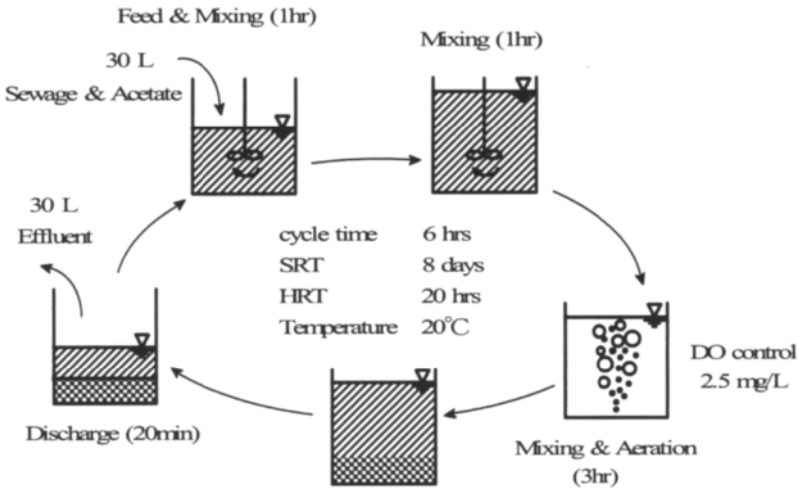


Figure 1 Cyclic operation of SBR for nutrient removal

sludge process treating municipal wastewater. In each cycle, 30-L of the municipal wastewater supplemented with acetate (40 mg-COD/L) was added to the reactor with the effective volume of 100-L. After settling, the same amount of supernatant was withdrawn, corresponding to HRT of 20 hours. The operating temperature and the DO level of aeration phase were controlled at 20° C and 2.5 mg/L, respectively. Excess sludge was wasted at the end of aeration phase of each cycle by drawing mixed liquor of 3.1-L, which corresponded to SRT of 8 days.

Acetate was added to actual sewage so that stable EBPR could be obtained under fluctuating influent conditions. After obtaining stable EBPR in SBR for two months, addition of acetate was reduced to 20 mgCOD/L for 16 days and then it was stopped. Influent and effluent qualities were monitored during the perturbation.

2.2. Sampling and analysis

Influent was collected from its storage tank at 4 ° C every three hours by an auto-sampler. Daily composite sample was subjected to water quality analysis such as PO_4-P, NH_4-N, NO_2-N and NO_3-N. Effluent nitrogen and phosphorus concentrations were also measured to investigate nutrient removal performance. Sludge samples were collected from the reactor at the end of aerobic phase and were subjected for quinone profile analysis described below. In addition, sludge samples were taken from a laboratory size A/O SBR fed with acetate, membrane separation bioreactor (MBR), and circulated denitrification reactor (CDR). The latter two processes were operated at our pilot plant installed at a municipal sewage treatment.

2.3. Quinone analysis

Sludge sample was mixed with three volumes of a chloroform-methanol mixture (2:1, v/v) and homogenized for 2 min. After centrifugation (7,000 rpm, 10 min), the resulting aqueous phase was removed, while the lipid phase that contained quinone was recovered. The extraction procedure was repeated three times with the chloroform-methanol and all extracts were concentrated in a vacuum and re-extracted with n-hexane.

Thereafter, the crude quinone extract in n-hexane was concentrated by Sep-Pak Silica cartridge and separated to MK and Q with 2 % and 10 % diethylether-hexane, respectively [10]. Quinone components were analyzed by high performance liquid chromatography and then identified by spectro-chromatography. Equivalent number of isoprene unit (ENIU) was calculated from their retention time [7]. Identified quinone components, Q and MK with n isoprene units, are expressed as Q-n and MK-n, respectively.

2.4. Phosphorus release test

To evaluate PAO content in activated sludge, P release test was carried out under anaerobic condition with addition of acetate. Centrifuged sludge sample was mixed with oxygen-free dilution medium for BOD analysis in which sodium acetate (140mgC/L) and sodium bicarbonate as buffer (200mg/L) were added. The mixture was stirred for 6 hours at 20° C under anaerobic condition. Sample was collected every 30 minutes and subjected to phosphate analysis. To avoid oxygen contamination in the mixture, N_2 gas was purged during sampling. Change of released P concentration was used to estimate PAO concentration by curve fitting method based on the P release kinetic equation in the ASM2.

3. RESULTS AND DISCUSSIONS

3.1 Treatment performance of SBR

Figure 2 shows changes of influent quality and MLVSS during the experimental period. Influent total CODcr concentration varied from 60 to 265 mg/L. Low CODcr values were observed after rainfall events on Day 1 and Day 12. Similar variation is shown in influent total N concentration. With respect to sludge parameters, reduction of added acetate caused corresponding decrease in biomass concentration, while there was no bulking problem during the experiment. However, unfortunately, a large amount of sidestream wastewater from sludge treatment was introduced to the influent line on Day 22. Therefore, we stopped the experiment because of significant change in sludge characteristics in the SBR.

Figure 3 shows changes of effluent N and P concentrations. As we expected, sufficient nitrification took place during the whole perturbation period. On the contrary, P removal deteriorated from Day 2 when influent total CODcr decreased to 60 mg/L. Effluent phosphate concentration reached more than 1 mg/L and then gradually decreased until Day 13. The second rainfall event on Day 12 caused low influent CODcr, which also caused deterioration of P removal. When the acetate addition was ceased on Day 16, effluent P concentration was over 1 mg/L and then became higher on Day 18.

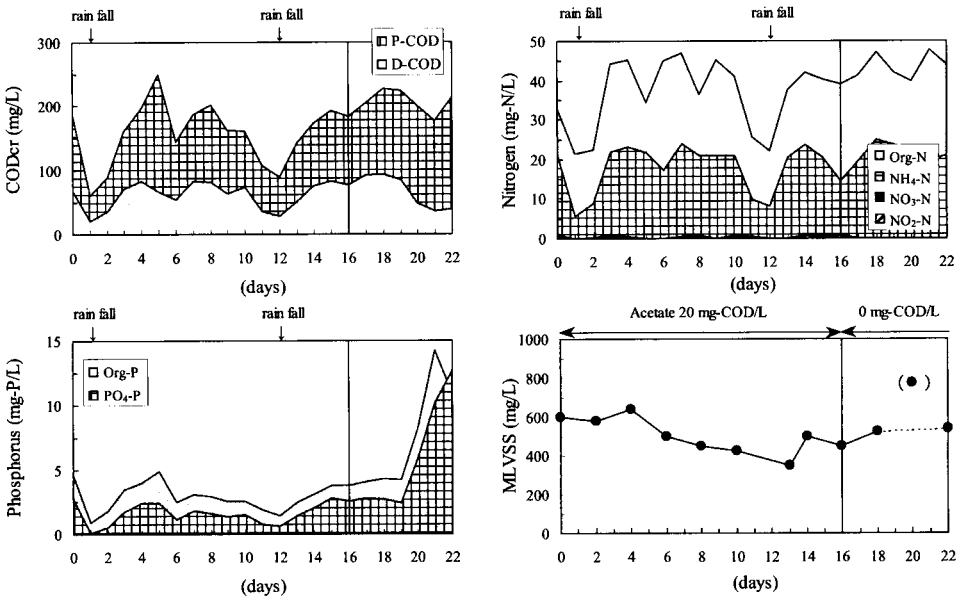


Figure 2 Changes of influent sewage quality and MLVSS

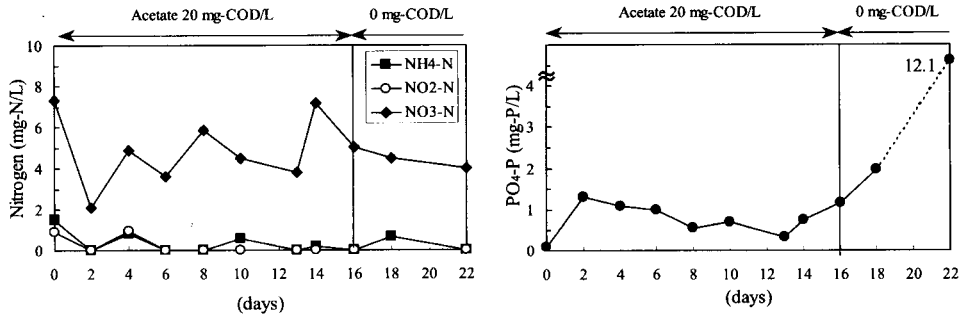
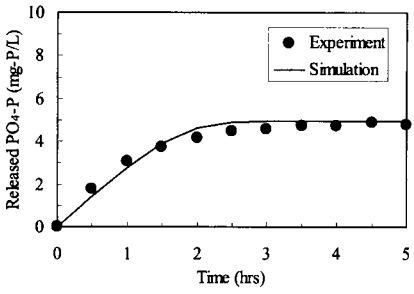


Figure 3 Changes of N and P concentrations in effluent

3.2. Evaluation of PAO content in activated sludge

We carried out P release tests for sludge collected at the end of aerobic phase. Rapid and higher P release implies higher PAO concentration and more accumulated polyphosphate in sludge. In the ASM2, phosphorus release rate is expressed by the equation in Figure 4.

Values of stoichiometric and kinetic parameters in the rate equation were given according to typical values proposed in the IAWQ technical report. X_{PP}, S_A, and X_{PAO} are time-variables. Since added acetate concentration was much higher than saturation constant for acetate (K_A), the term of acetate was regarded as unity. X_{PP} at time=0 was estimated using finally released P concentration in each test. Eventually, X_{PAO} was obtained as COD base by fitting model simulation to experimental data using nonlinear least square method. Figure 4 shows an example of curve fitting for the results on Day 13.



$$r_{P\text{-release}} = Y_{PO4} \cdot q_{PHA} \cdot \frac{S_A}{K_A + S_A} \cdot \frac{X_{PP}/X_{PAO}}{K_{PP} + X_{PP}/X_{PAO}} \cdot X_{PAO}$$

$r_{P\text{-release}}$: P-release rate
Y_{PO4}: Poly-P requirement for PHA storage
q_{PHA}: rate constant for storage of PHA
S_A : acetate concentration
K_A : saturation constant for S_A
X_{PP}: stored Poly-P in PAO
K_{IPP}: inhibition coefficient for X_{PP} storage
X_{PAO}: PAO concentration

Figure 4 Phosphorus release test on Day 13

Based on results of P release tests, PAO concentration and its content were estimated as shown in Figure 5. The COD concentration of PAO was converted to MLVSS value using a factor of 1.416 mgCOD/mgCell-VSS. The initial PAO concentration was estimated as 86 mgVSS/L, which corresponds to about 15% of total MLVSS in the sludge. There was a rapid decrease in PAO concentration on Day 2. Once it slightly increased on Day 4, but then gradually decreased until Day 16. After stopping the acetate addition, the PAO level was around 40 to 50 mg/L. Since MLVSS concentration also decreased during the perturbation, the PAO content showed a different behavior from Day 2 to Day 16. Because decreasing rate of PAO concentration was smaller than that of MLVSS, the content increased from Day 4 to Day 13. The increment has a good agreement with decreasing effluent P concentration during the period. PAO content decreased to 8% of MLVSS from Day 16 to Day 22, because acetate addition was stopped. The estimated PAO content became half during the experiment.

3.3. Quinone profile and microbial community change in SBR

Figure 6 shows the change in mole fraction of quinone from Day 4 to Day 20. Initial mole fraction is not shown due to a technical trouble in analysis of menaquinone on the starting day. For all samples, the most predominant ubiquinone was Q-8 in the sludge. The second and third were Q-10 and Q-9, respectively. The predominance order of menaquinone was MK-8 (H4) and MK-7. These results were similar to data reported by Hiraishi *et al.* (1998)[8] on activated sludge in AO process plants.

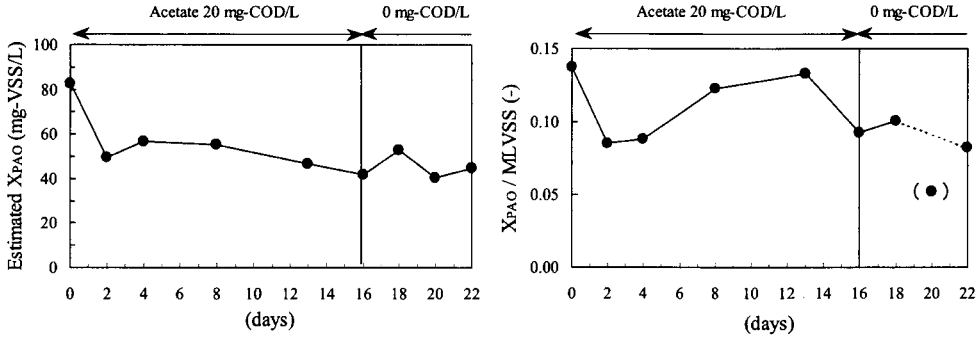


Figure 5 Changes of estimated PAO concentration and its content

To investigate microbial community change during the experiment based on quinone profile data, dissimilarity index value (D-value) was calculated according to the Eq. (1).

$$D(i,j) = 0.5\sum_{k=1}^{n}\left|x_{ik} - x_{jk}\right| \qquad (1)$$

where n is the number of quinone components and x_{ik} and x_{jk} are the fractions of the k quinone component for the i and j samples, respectively.

D-value is in the range of 0 to 1. When the value is not more than 0.1, microbial communities of two samples are similar. Values more than 0.2 are interpreted as a significant difference of community [11]. Since the values were no more than 0.1 among all samples as shown in Table 1, no significant shift of microbial community took place during the experiment.

3.4. Relationship between PAO content and quinone fractions

Although there was no significant change in the quinone profile, estimated PAO content changed dynamically after the reduction of added acetate. Relationship between PAO content and fractional change of quinone species was investigated in order to obtain information for identification of any specific quinone indicator to PAO.

Panels of Figure 7 show fractional changes of 3 ubiquinone and 7 menaquinone species. PAO content change is also shown in the same fraction range of vertical axis. Since change of PAO content in the activated sludge was just within the range of 5% during the experiment, the loss of PAO may not reflect a significant change in quinone fraction. However, if they

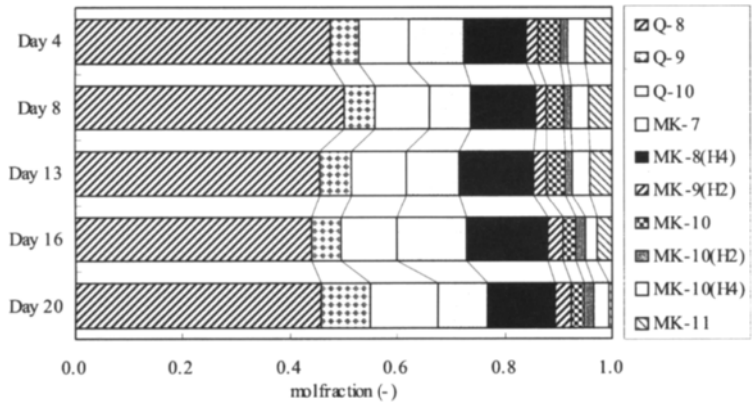


Figure 6 Change in quinone profile of activated sludge in EBPR-SBR

Table 1
Dissimilarity analysis

	Day 8	Day 13	Day 16	Day 20
Day 4	0.04	0.04	0.08	0.09
Day 8	-	0.05	0.10	0.09
Day 13	-	-	0.05	0.07
Day 16	-	-	-	0.09

have a predominant quinone species that is not a quinone fraction of major bacterial groups, there should be a similar variation in the corresponding quinone to changing pattern of PAO content. In our previous study [12], we found that fractional change of two menaquinone species (MK-7 and MK-8(H4)) had good agreement with increased P uptake rate at aerobic phase during a start-up of EBPR in SBR process. These two species were considered as possible indicators for development of EBPR activity.

The variation pattern of MK-8(H4) and MK-7 look relatively similar to that of PAO content in this experiment, but they increased on Day 16 while PAO content decreased. The other fractional changes have clearly different trend from that of PAO content. Therefore, we concluded that the investigation did not give a clear relationship between kinetically estimated PAO content and each quinone fraction. In this study PAO concentration was estimated based on kinetic model. There might be a limitation in comparison of kinetically estimated PAO content and analytical data on biomarker such as quinone profiles.

Among the variations of 10 quinone species, several similar patterns were found. For example, Q9 and Q10 simply increased by the reduction of added acetate, while MK-10 and MK-11 simply decreased. MK-7 and MK-8(H4) had a relatively similar fluctuation pattern. Although there was no clear relationship between PAO content and quinone species, the reduction of added acetate caused interesting change in quinone fractions. In the previous study on start-up of anaerobic/aerobic SBR [12], acetate was added to influent sewage to promote the EBPR. In that experiment, Q-9 and Q-10 simply decreased under acetate addition condition, which is opposite to the trend under acetate reduction condition as above-mentioned. MK-10 and MK-11 had a very similar behavior each other. MK-7 and MK-8(H4) had also a relatively similar pattern. The corresponding pattern change for both cases of reduced and added implies that the set of the quinone species seems to be specific to acetate metabolism by bacterial group.

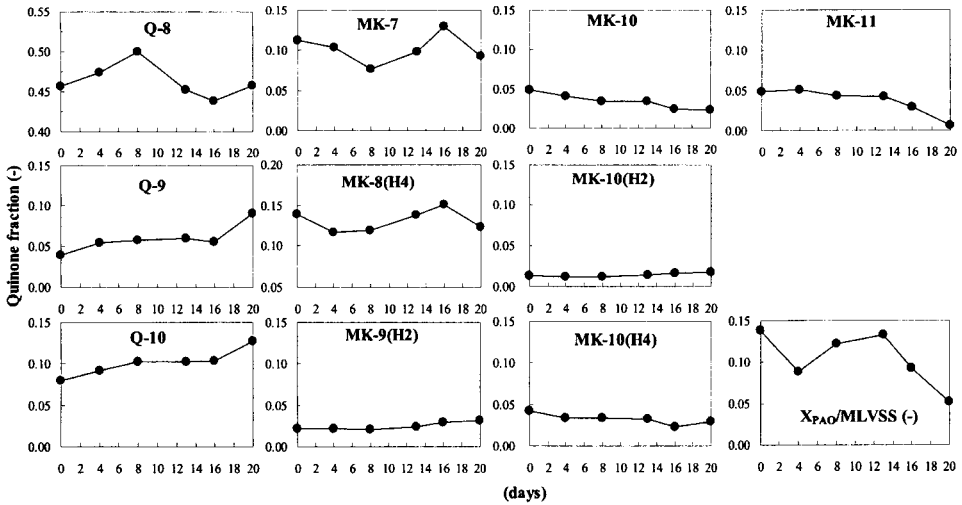


Figure 7 Fractional changes in quinone content

3.5. Comparison of quinone profiles with other activated sludge

In order to compare the quinone profiles of the SBR sludge with those of other activated sludge under different operation, sludge samples were taken from other reactors. The reactor types and their operations are summarized in Table 2. Another SBR process (SBR(B)) was also operated under anaerobic and aerobic condition, while MBR and CDR were not EBPR processes. MBR had a very long SRT condition and CDR was operated for denitrification.

Figure 8 shows quinone profiles of sludge in the above-mentioned reactors. Among them, the profile for SBR (B) fed with acetate was much different from the other processes that treated same actual sewage. The difference was quantitatively shown by the dissimilarity index values more than 0.2 between SBR (B) and others. High dissimilarity comes from difference in ubiquinone (Q) content and poor diversity of menaquinone (MK) in SBR (B) sludge. Single supply of acetate seems to make microbial community in activated sludge simple. Higher content of Q-9 in activated sludge was also reported by Okada *et al.* (1992)[13]. In their operation, acetate was a main substrate for the SBR process.

On the contrary, MBR and CDR have a relatively low D value of 0.127 under extremely different SRT conditions. As reported by Hiraishi *et al* (1998)[8], substrate or influent condition had more influence on quinone profiles than operational condition.

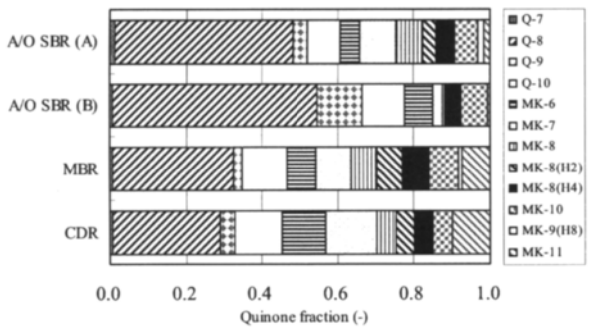
4. CONCLUSIONS

A bench scale anaerobic/aerobic sequencing batch reactor (SBR) was operated with feed of actual sewage to investigate the population dynamics using quinone profile analysis. Quinone profiles showed that Q with 8-isoprene unit (Q-8) was present as the most predominant, Q-10 and Q-9 were the second and the third. The most predominant MK was MK-7, when stable and good EBPR was achieved by addition of acetate to influent sewage.

Table 2
Influent and operational conditions of reactors for comparison

Reactor	A/O SBR (A)	A/O SBR (B)	MBR[1)]	CDR[2)]
Influent	Sewage + Acetate	Acetate	Sewage	Sewage
SRT	8 d	10 d	> 100 d	10 d
HRT	20 h	10 h	36 h	6 h

1) Membrane separation Bio Reactor
2) Circulated Denitrification Reactor



Dissimilarity index value

	A/O SBR (B)	MBR	CDR
A/O SBR (A)	0.206	0.184	0.237
A/O SBR (B)	-	0.314	0.353
MBR	-	-	0.127

Figure 8 Comparison of quinone profiles in different activated sludge

The reduction of added acetate and the dilution of influent sewage due to rainfall on Day 1 caused rapid deterioration of EBPR and loss of PAO concentration. Estimated PAO content in sludge changed from 14% to 8% in the experiment. The reduction of added acetate caused a large impact to loss of PAO and fractional change in quinone profile during the experiment. However, the dissimilarity analysis revealed that there was no so significant change in microbial community shift. In other words, change in microbial community was evaluated as medium level based on quinone profile analysis. Relationship between changes in quinone fraction and PAO content was compared. Although fluctuation of two menaquinone species (MK-8(H4) and MK-7) looked relatively similar to change of PAO content, there was no clear relationship between them.

The comparison of temporal variation of each quinone species would be informative to evaluate microbial community change. There were several sets of quinone species that had similar response to reduction and addition of acetate in anaerobic/aerobic SBR process. In addition, the quinone profiles of the SBR sludge were also compared with those of activated sludge in other processes that were not designed for EBPR. The profile for the SBR fed with acetate was much different from the other processes that treated same actual sewage. High dissimilarity comes from difference in ubiquinone (Q) content and poor diversity of menaquinone (MK) in acetate-fed sludge. Supply of acetate to EBPR system could not represent complex microbial community in EBPR sludge treating actual sewage.

REFERENCES

1. Mino, T., M.C.M. van Loosdrecht, and J.J. Heijnen (1998) Wat. Res., 32, 3193-32071.
2. Henze, M., Grady Jr, C.P.L. Gujer, W., Marais,G.V.R, and Matsuo,T. (1986) Activated Sludge Model No. 1., IAWPRC Scientific and Technical Report No.1, IAWPRC
3. Oles, J., and Wilderer, P.A. (1991) Wat. Sci. Tech. 23(4), 1087-1095
4. Henze, M., Gujer, W., Mino, T., Matsuo,T., Wentzel, M.T., and Marais,G.V.R,. (1995) Activated Sludge Model No. 2. IAWQ Scientific and Technical Report No.3, IAWQ
5. Gujer, W. Henze, M., , Mino, T., and van Loosdrecht, M.C.M. (1999) Wat. Sci. Tech, 39, 138-194
6. Furumai, H., Kazmi, A.A., Fujita, M., Furuya, Y., Sasaki, K., (1999) Water Research, 33, 2708-2714.
7. Hiraishi, A., Masamune, K. and Kitamura, H. (1989) Appl. Environ. Microbiol., 55, 897-901.
8. Hiraishi, A., Y. Ueda, and J. Ishihara (1998): Appl. Environ. Microbiol. 64, 992-998
9. Sudiana, I.M., Mino, T., Satoh, H., and Matsuo, T. (1998) Wat. Sci. Tech., 38(8/9), 69-76
10. Fujie, K. Hu, H-Y., Tanaka, H. and Urano, H. (1994) Wat. Sci. Tech, 29, 373-376
11. Hiraishi, A., Morishima, Y., Takeuchi, J. (1991) J. Gen. Appl. Microbiol., 37, 57-70
12. Fujita, M., Chen, H., and Furumai, H. (1999) Proc. 7th IAWQ Asian-Pacific Reg. Conference, Taiwan, Vol.1 , 499-504
13. Okada, M., Lin, C.K., Katayama, Y., and Murakaki, A. (1992) Wat. Sci. Tech,., 26 (3/4), 483-491

Applicability of FISH, dot blot hybridization, antibody immobilized latex coagulation, and MPN techniques as enumeration methods for ammonia-oxidizing bacteria in various water environments

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Fluorescent *in situ* hybridization (FISH), dot blot hybridization, antibody immobilized latex coagulation, and MPN methods for enumeration of ammonia-oxidizing bacteria were applied to various water environments, namely pure culture, enrichment cultures, mixed liquors and effluents of municipal wastewater treatment plants (WWTPs), and river water. The samples were categorized and applicability of these methods at the present time was discussed. Higher applicability of dot blot hybridization and underestimation caused by MPN were demonstrated. Antibody immobilized latex coagulation could be easily applied to the samples with less impurity particles. Successful application of FISH was limited to high ammonium loading systems due to weak signal, auto-fluorescence, and non-specific binding.

1. INTRODUCTION

Enumeration of ammonia-oxidizing bacteria (AOB) is useful in control of wastewater treatment processes, because AOB works as one of the key elements in biological nitrogen removal. Till lately, enumeration of AOB has been achieved only by culture-dependent MPN (most probable number) methods [1], which have some fundamental defects such as long culturing time (1 month) and difference of culturability among the species of AOB [2].

Recently, development of biology and molecular microbiology enabled us to apply antibody methods [2, 3], fluorescent *in situ* hybridization (FISH)[4, 5] and dot/slot blot hybridization methods [5, 6] using 16S rRNA targeted oligonucleotide probe for enumeration of AOB. Taking advantage of these methods, enumeration of AOB may be applied to not only wastewater treatment system but also other aquatic systems such as river, lake, sea, and/or sediment. These enumerations in lower concentration range will

help us to understand the nitrogen cycle in the environment more precisely and assess the risk potential of oxygen consumption by AOB. Moreover, in future, it might be possible that artificial environmental impacts will be monitored by AOB's profiles, because AOB is thought to be ubiquitous in the environment and sensitive to environmental stresses.

The FISH method has been reported successfully for biofilm systems [7-9] and high ammonium loading systems [4, 10], however, for relatively low ammonium loading water environment systems, such as municipal wastewater treatment processes and natural water bodies like river, AOB enumerations have not been reported well. As for dot/slot blot hybridization, very few studies have been known about enumeration of AOB in the environments.

This research executed practical enumerations of AOB for pure culture, enrichment cultures, municipal wastewater treatment processes and its effluents, and river water by the FISH, dot blot hybridization, antibody immobilized latex coagulation, and MPN methods. Applicability of each method was discussed.

2. MATERIALS AND METHODS

2.1. Samples and pre-treatment

Pure culture and enrichment cultures. Nitrosomonas europaea (IFO14298) was selected as pure culture of AOB. Growth medium and condition designated by IFO (Institute for Fermentation, Osaka) was used for culturing. Two enrichment (inorganic and organic) cultures of AOB with high ammonium loading were also cultured.

Lab-scale reactor. A lab-scale reactor was operated with an artificial wastewater. Peptone was the only nitrogen source in this system. Though allylthiourea (ATU) was constantly added, ammonia-oxidizing activity was observed in this reactor.

Mixed liquors and effluents of municipal wastewater treatment plants (WWTPs). Mixed liquors and effluents were collected from two municipal WWTPs (X, Y). Both plants were operated as conventional activated sludge system. Plant Y was equipped with sand filtration system for the effluent polish-up. In both systems, ammonia-oxidizing activity was observed. Mixed liquors were collected at the end of aeration tanks. Effluents were collected from sedimentation tanks before and after chlorination.

River water. River water of the Tama River in the Tokyo area was sampled from shorefront at the following four sampling points: from the upstream, Hamura, Sekido, Koremasa, and Maruko.

Pre-treatment. Effluents from municipal WWTPs and river water were 300 times concentrated by centrifugation (about 3000g, 30 minutes x2, 4°C) for enumeration methods other than MPN.

2.2. Enumeration methods

Most Probable Number (MPN). The MPN method was performed based on Sewage Testing Methods [11]. The samples were diluted into 10-fold dilution series and cultured in

five tubes for each dilution steps. The culturing temperature was set at 28°C and the culturing period was 1 month. Nitrite production by AOB was detected by the GR (Griess-Romijn) reagent and the concentration of AOB was estimated by the MPN table.

Antibody immobilized latex coagulation. A simple and ready-to-use detection kit for nitrifying bacteria "Immuno latex "Kenshutsukun"" (Yakult Central Institute for Microbiological Research, Yakult Honsha Co., Tokyo, Japan) was used. Polyclonal antibody for AOB combined with latex particles was provided as a solution in this kit. After making 2-fold dilution series, the diluted samples were dropped on a microtiter plate, and the antibody-latex reagent was dropped on the samples. Subsequent to incubation for 4-30 hours at room temperature, AOB was detected by latex coagulations. Enumeration was achieved by the following equation: (Concentration of AOB) = (Maximum dilution times where coagulation can be detected) x (Titer value of the antibody provided by the kit).

Fluorescent in situ hybridization (FISH). The FISH method was performed on the protocols described by Amann (1995) [12], Wagner *et al.* (1995) [4], and Mobarry *et al.* (1996) [5]. All the samples were fixed by 3% paraformaldehyde solution for more than 2 hours at 4°C, after ultrasonic dispersion for the samples needed, spotted on gelatin-coated slide glasses. Samples on slide glasses were dehydrated by soaking in ethanol series (50%, 80%, and 98%) each for 3 minutes. Hybridization buffer was made from 0.9M NaCl, 20mM Tris/HCl, 0.01% SDS, and formamide (concentration shown in Table 1), pH 7.2; in addition to that, 20% Block Ace (Dainippon Pharmaceutical, Tokyo, Japan) was added in order to inhibit non-specific binding of probe with impurity particles. Fluorescent-labeled oligonucleotide probes were dissolved with the hybridization buffer, and hybridized with the samples for 2 hours at 46°C. The probes used for FISH are listed in Table 1. Detection of AOB was done by the Nso190 probe that is specific for most of AOB. However, because of weak signal in the observational results using Nso190, experiments using the NEU probe, which is specific for Genus *Nitrosomonas* and gives stronger signal than Nso190, were executed in parallel with Nso190 experiments. Moreover, the EUB338 probe specific for most of all eubacteria was used for easier recognition of target cells and the NON probe that does not hybridize to any organisms was also used to distinguish non-specific binding of probe with impurity particles. These probes were added simultaneously with Nso190 or after NEU. After the hybridization, washing was done by the buffer without formamide (20mM Tris/HCl, 0.01% SDS, and NaCl concentration shown in Table 1, pH 7.2) for 20 minutes at 48°C. After applying SlowFade-Light (Molecular Probes, Eugene, Ore., USA) as anti-fading reagent, sample observation and image acquisition were executed by TCS-NT confocal laser scanning microscope (Leica Microsystems Heidelberg, Germany) equipped with Ar/Kr laser. Randomly 10 images were obtained for one sample. After the image processing, the probe-hybridized cells were counted manually. The image processing was achieved by Scion Image for Windows (Release Beta 3, Scion Corporation, Frederick, Md., USA, free software available at http://www.scioncorp.com/).

Table 1
Oligonucleotide probes used for the FISH method in this study

Probe	%FA	NaCl	Label	Specificity	Reference
Nso190	25†	105	XRITC	Ammonia-oxidizing β-*Proteobacteria*	Mobarry *et al.*, 1996 [5]
NEU	40	25	XRITC	Genus *Nitrosomonas*	Wagner *et al.*, 1995 [4]
EUB338	20	171	FITC	Most of all *Eubacteria*	Amann *et al.*, 1990 [13]
NON	20	171	Cy5	Does not hybridize to any organisms	Wallner *et al.*, 1993 [14]

*%FA: formamide concentration (%) in hybridization buffer, NaCl: NaCl concentration (mM) in washing buffer.

† Formamide concentration of Nso190 probe was re-optimized by Konuma *et al.*, 2000[15].

Dot blot hybridization. RNA was extracted by the AGPC (Acid Guanidinium Phenol Chloroform) method using TRIZOL reagent kit (GIBCO BRL, Life Technologies, Frederick, Md., USA). Hybridization and detection of oligonucleotide probes were achieved by DIG system (Boehringer Mannheim Biochemica, Mannheim, Germany) according to the manufacturer's protocol. Extracted RNA was diluted into 3-fold dilution series, spotted manually on positively charged nylon membranes (Boehringer Mannheim), and crosslinked in GS Gene Linker UV chamber (Bio-Rad Laboratories, Calif., USA). DIG-labeled Nso190 probe was hybridized for more than 6 hours at 50°C. DIG Easy Hyb (Boehringer Mannheim) was used as hybridization buffer. After washing, the probe was detected by enzyme-catalyzed color reaction using DIG Nucleic Acid Detection Kit (Boehringer Mannheim). The Nso190 enumerations of the pure culture of *Nitrosomonas europaea* by the FISH method were used as references for the enumerations of the samples.

3. RESULTS AND DISCUSSION

FISH, dot blot hybridization, antibody immobilized latex coagulation, and MPN methods for enumeration of AOB were applied to the pure culture and the enrichment cultures of AOB, the lab-scale reactor, the mixed liquors and the effluents of the municipal WWTPs, and the river water in order to assess the applicability of these methods. Because the MPN method has been conventionally used for enumeration of AOB, experimental results in Figure 1 are compiled in the style of comparison based on the results obtained by the MPN method.

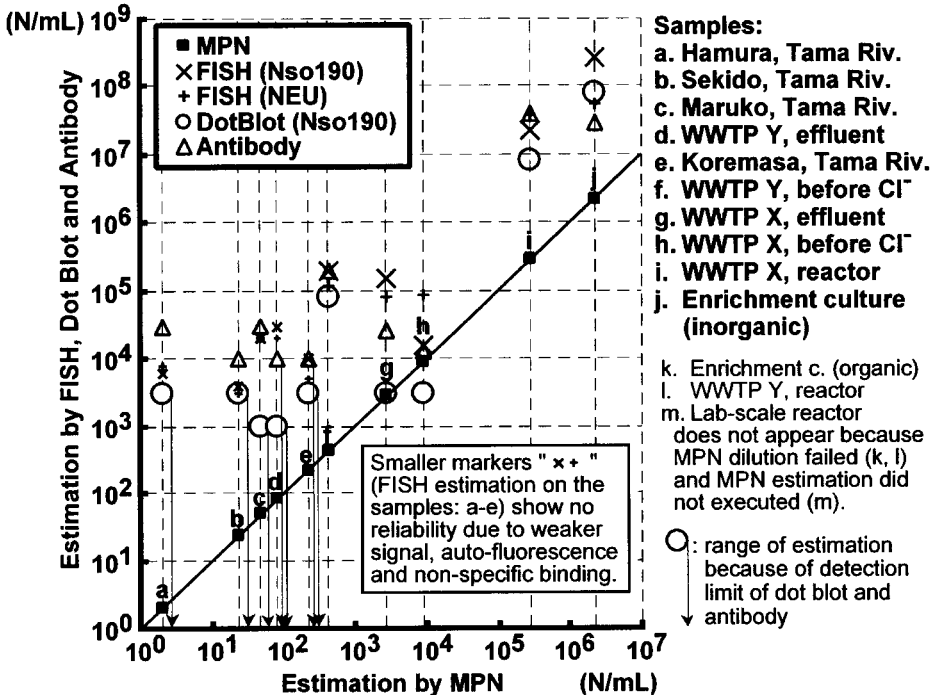


Figure 1. Comparison of enumeration results by FISH, dot blot hybridization, antibody immobilized latex coagulation, and MPN (based on the results of MPN). WWTP: Wastewater treatment plant.

3.1. The problematic MPN method

Most of the MPN enumerated values were lower than the other methods by 1-2 order(s) in Figure 1. Indeed the other methods faced the detection limit and/or trouble in the enumerations in the lower concentration range, but enumerations by the other methods were quite successful in higher concentration such as enrichment culture, therefore the MPN enumeration was thought to underestimate the concentration of the targeted cells. This observation agreed with Araki *et al.* (1999)[8] and Belser (1970)[2]. Cluster formation of AOB (Figure 2) and unculturable AOB are likely responsible to this phenomenon. Although only MPN can give reasonable results owing to its lower detection limit (7 x 10^{-4}/mL when concentrated by 300 times) when AOB concentration is low, the enumeration results should be treated carefully because of the defects above.

3.2. Applicability of each method for sample categories

As for the enrichment cultures, the FISH method was most successful among all the enumerations in this study. Fluorescence from the probe-hybridized cells was much more brighter than auto-fluorescence (background fluorescence), furthermore non-specific

binding of probe with impurity particles was less than other samples (Figure 2). Enumerations were conducted without any doubt. Counting of dead cells could not be completely denied, but from the fact that the number of Nso190 stained cells were about 25 - 50% of total cells stained by YOYO-1 in the pure culture enumeration [16], low activity cells and dead cells were thought to be excluded from the FISH enumerations. On these samples, enumeration results by FISH, dot blot hybridization, and antibody immobilized latex coagulation agreed well within 1 order. Thus these methods were judged to be practically applicable to enrichment cultures.

As for the mixed liquors and the effluents from the WWTPs, no trouble was experienced in dot blot hybridization. In the case of FISH enumeration, however, fluorescence from probe-hybridized cells was weak and discrimination of probe fluorescence from auto-fluorescence was often difficult. Moreover non-specific binding of probe with impurity particles was a serious problem. Simultaneous hybridization with the EUB338 + NON probe and image processing technique to remove auto-fluorescence and non-specific binding (Figure 3 [16]) were developed to overcome these problems with FISH, but they were still not enough. Not only the Nso190 probe, but also the NEU probe, which usually produces brighter image than Nso190 if it hybridizes to the target cells, was used in the same way, but this trouble was not cleared. In the case of antibody immobilized latex coagulation kit, non-specific binding was also observed, but it was less serious than FISH.

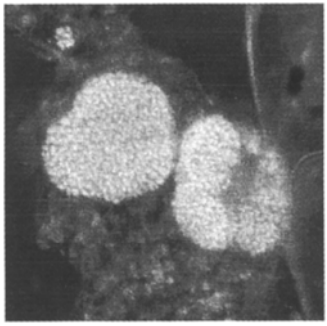
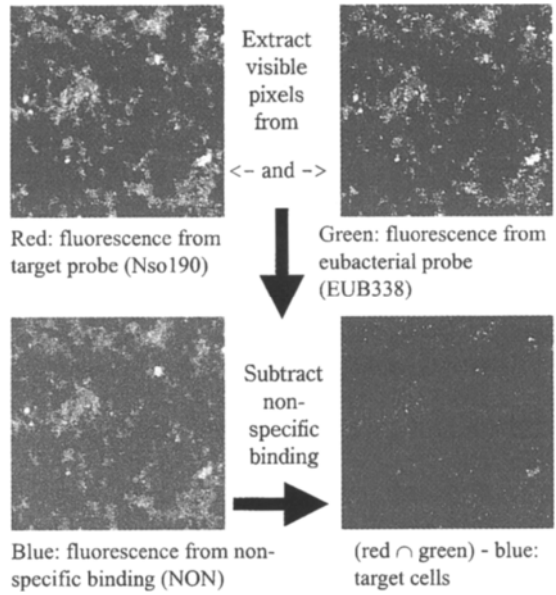


Fig. 2. Cluster of AOB observed in the organic substrate enrichment culture by the FISH method. Fluorescence was bright enough for enumeration.



Considering simplicity of this kit, this result is notable. This matter can be explained by that this kit was developed and optimized for actual wastewater treatment processes. It seems that the applicability of this kit depends on the amount of impurity particles in samples. Therefore in these kinds of samples, dot blot hybridization was thought to have high reliability, but reliability of FISH and antibody immobilized latex coagulation was thought to be relatively low. Considering this viewpoint, Figure 1 can be re-drawn as Figure 4 based on the results of dot blot hybridization.

In Figure 4, large underestimations by MPN, occasional large overestimations by FISH and overestimations by antibody immobilized latex coagulation are observed. Because of the facts above, dot blot hybridization is supposed to be recommended among these methods. However, we should keep in mind that dot blot hybridization has potential problems as follows: biases from RNA extraction and difference of rRNA content between AOB in environmental samples and pure culture.

As for river water samples, dot blot hybridization could not detect the target RNA due to its detection limit. Reasonable AOB enumeration by FISH was almost impossible because of weak target fluorescence, auto-fluorescence, and non-specific binding, and also impossible by antibody immobilized latex coagulation because of non-specific binding. Enumerations were executed compulsively. Indeed true concentration of target cells may

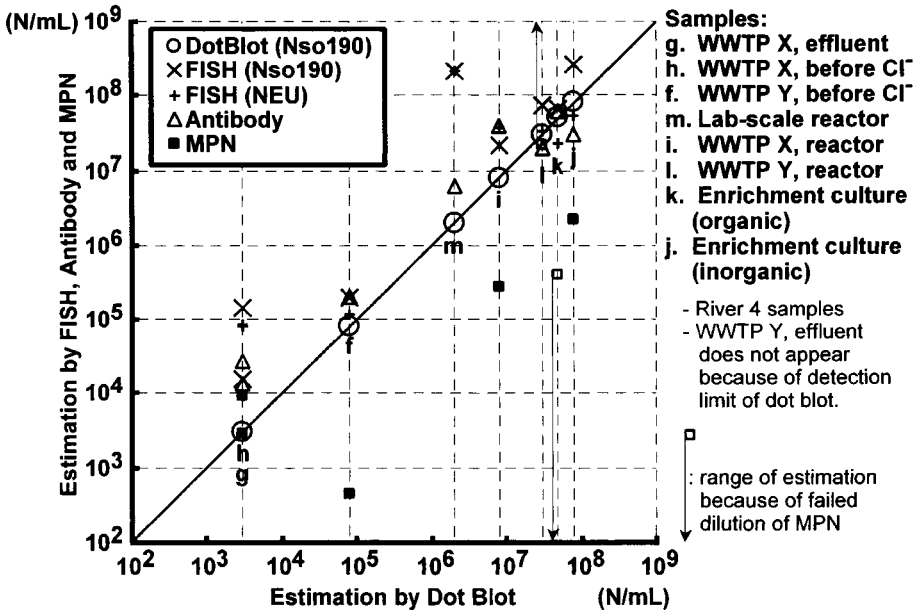


Figure 4. Comparison of enumeration results by FISH, dot blot hybridization, antibody immobilized latex coagulation, and MPN (based on the results of dot blot hybridization).

Table 2
Classification of recommendable methods by sample categories

	Pure culture Enrichment culture	WWTP mixed liquor	WWTP effluent	River water
Properties	Impurity particles are few and target cells are dominant.	Amount of impurity particles depends on the nature of samples. Target cells are not always dominant.	Many impurity particles exist. Cell concentration is low.	Many impurity particles exist. Cell concentration is much lower than the other samples.
Dot Blot Hybridization	+. Quantifiability is thought to be robust, though the experimental procedures are not easy. It takes about 3 days for one experiment.			---. Enumeration cannot be executed.
FISH	++. Quantification is quite easy owing to the bright fluorescence. Reliability is thought to be very high. It takes about 1.5 days for one experiment.	-. Quantification is difficult due to the dark fluorescence. Enumeration can be executed, however, reliability is doubtful.	--. Quantification is difficult due to dark fluorescence, auto-fluorescence, and non-specific binding. Enumeration may be executed compulsively, however, reliability is very doubtful.	
Antibody immobilized latex coagulation	+. Quantification is easy and the experimental procedures are quite easy. It takes about 30 minutes for the experimental procedure and about 1 day for waiting. Although non-specific binding is observed, the enumeration is thought to be reliable.		-. Enumeration can be executed, however, reliability is doubtful because of non-specific binding.	---. Enumeration may be executed compulsively, but reliability is very doubtful because of non-specific binding.
MPN	--. The experimental procedures are easy and enumeration can cover the lower target concentration, however, underestimation may occur. It takes about 1 month for waiting.			-. Enumeration can be done only by this method.

†++, +, -, --, ---: Level of recommendation:
++ : Fully recommended, enumeration has high reliability.
+ : Recommended, though still some points for discussion remain.
- : Enumeration may be done, however, reliability is low.
-- : Not recommended. Enumeration may be done, however, reliability is very low.
--- : Not recommended. Enumeration cannot be executed.

be under detection limits of these two methods (FISH: 9 x 10^3/mL and antibody: 1 x 10^4/mL when concentrated by 300 times), but the detection limits were not serious in these cases because enumerations (and overestimations) were actually done. Only MPN can give enumeration results constantly (Figure 1), however due to the reasons above, the true value is supposed to exist between MPN enumeration and detection limit of dot blot hybridization (1 x 10^3/mL when concentrated by 300 times). For low concentration samples, dot blot hybridization will be applicable if the concentration factor is more increased. Furthermore, improvement of the FISH method, such as fluorescence enhancement, application of *in situ* PCR or PNA (peptide nucleic acid) hybridization, may become a breakthrough of the FISH method.

The ratings at the present time about the applicability of these methods mentioned above are categorized and summarized as Table 2. Nature of samples is also described and five levels of recommendation are proposed.

4. CONCLUSION

FISH, dot blot hybridization, antibody immobilized latex coagulation, and MPN methods were applied to the samples from various water environments. The samples were categorized and the advantages and disadvantages of these methods at the present time were discussed and summarized. MPN enumeration appeared to be problematic. Dot blot hybridization was found to be a relatively reliable method for AOB enumeration. Antibody immobilized latex coagulation could be easily applied to the samples with less impurity particles. FISH can be successfully applied to high ammonium loading systems, but weak signal, auto-fluorescence, and non-specific binding can cause problems when applied to low ammonium loading systems. Improvement of techniques will be needed for FISH.

REFERENCES

1. Matulevich V. A., Strom, P. F., and Finstein, M. S. (1975). Appl. Microbiol., 29, 265-268.
2. Belser L. W. (1979). Ann. Rev. Microbiol., 33, 309-333.
3. Sandén B., Grunditz C., Hansson Y., and Dalhammar G. (1994). Wat. Sci. Tech., 29(7), 1-6.12.
4. Wagner M., Rath G., Amann R., Koops H. P., and Schleifer K. H. (1995). Appl. Microbiol., 18, 251-264.
5. Mobarry B. K., Wagner M., Urbain V., Rittmann B. E., and Stahl D. A. (1996). Appl. Environ. Microbiol., 62, 2156-2162.
6. Hovanec T. A., and DeLong E. F. (1996). Appl. Environ. Microbiol., 62, 2888-2896.
7. Schramm A., Beer D., Wagner M., and Amann R. (1998). Appl. Environ. Microbiol., 64, 3480-3485.

8. Araki N., Ohashi A., Harada H., and Machdar I. (1999). J. Japan Soc. Wat. Environ., 22, 152-159 (in Japanese).
9. Okabe S., Satoh H., and Watanabe Y. (1999). Appl. Environ., Microbiol., 65, 3182-3191.
10. Juretschko S., Timmermann G., Schmid M., Schleifer K. H., Pommerening-Röser A., Koops H. P., and Wagner M. (1998). Appl. Environ. Microbiol., 64, 3042-3051.
11. Sewage Testing Methods, Vol. A (1997). 1997 edn, Sewerage and Sewage Purification Department, City Bureau, Ministry of Construction / Water Supply and Environmental Sanitation Department, Environmental Health Bureau, Ministry of Health and Welfare (eds.) / Japan Sewage Works Association, Tokyo, Japan (in Japanese).
12. Amann R. I. (1995). In: Molecular Microbial Ecology Manual, A. D. L. Akkermans, J. D. van Elsas and F. J. de Bruijn (eds.), Kluwer Academic Publishers, Netherlands, section 3.3.6, pp. 1-15.
13. Amann R. I., Binder B. J., Olson R. J., Chisholm, S. W., Devereux R., and Stahl D. A. (1990). Appl. Environ. Microbiol., 56, 1919-1925.
14. Wallner G., Amann R., and Beisker W. (1993). Cytometry, 14, 136-143.
15. Konuma S, Satoh H, Mino T., and Matsuo T. (2000). Conference preprint, 1st world water congress of the International Water Association (IWA), Paris, book 3, 68-75.
16. Konuma S, Satoh H, Mino T., and Matsuo T. (1999). Environmental Engineering Research, Committee on Environmental Engineering, Japan Society of Civil Engineers (ed.), 36, 19-28 (in Japanese).

Nitrous oxide and nitric oxide emissions during sulfur denitrification in soil-water system

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Column and batch experiments were carried out to demonstrate the advantages of sulfur addition into soil as an electron donor for denitrification . When denitrification proceeded sufficiently, N_2O emission from the soil column packed with elemental sulfur was much lower than N_2O emitted from the actual paddy field. In the case of adding $CaCO_3$ for pH adjustment with inorganic sulfur, N_2O production was much suppressed. The NO emissions were much lower than N_2O emissions. Organic materials can promote nitrate removal though they may induce sulfate reduction. The sulfide formation had the inhibition of NO and N_2O reduction. Therefore, it is concluded that elemental sulfur is more appropriate additive to the soil-water system as an electron donor for denitrification than organic materials and FeS.

1. INTRODUCTION

Nitrous oxide (N_2O) is a significant atmospheric trace gas due to its high global warming potential, which is 280 times greater than that of CO_2[1]. According to IPCC (Intergovernmental Panel on Climate Change) report[1], the amount of N_2O in the atmosphere is currently increasing at a rate of 0.2 to 0.3% per year. Arai *et al.* [2] predicted a higher rate than this based on measurements of atmospheric N_2O in Japan. Therefore, urgent action to suppress N_2O emission is necessary.

Recently, increased anthropogenic nitrogen discharge to natural environments has resulted in nitrogen pollution in natural water bodies. Contamination of groundwater with nitrate-nitrogen is one form of this nitrogen pollution and a significant problem especially in agricultural areas where nitrogen loading is generally very high [3-6]. Nitrous oxide production in natural environments is predominantly caused by microbial activity, during nitrification[7] and denitrification[8] processes. During these processes, nitric oxide (NO), which contributes towards the destruction of stratospheric ozone layer, is also produced. N_2O and NO emissions are enhanced during the denitrification of the discharged nitrate-nitrogen[9]. Actually, our previous work showed the high fluxes of N_2O from the agricultural soil-water system contaminated with high nitrate-nitrogen [10].

An effective and feasible method to decrease N_2O and NO emissions, while removing nitrate-nitrogen sufficiently, by using denitrification activity of the soil-water systems should be explored. The addition of electron donor is one of the methods to improve the denitrification activity. But the results of the survey in the actual soil-water system indicated that high organic matters would facilitate N_2O emission[10]. On the other hand, inorganic materials, which generate autotrophic denitrification, seem to advance denitrification slowly and result in low N_2O emission.

Sulfur denitrification is based upon autotrophic denitrification by sulfur oxidizing bacteria such as *Thiobacillus denitrificans* and *Thiomicrospira denitrificans* [11-12]. The bacteria performing sulfur denitrification are listed in Table 1. Under aerobic conditions these bacteria use oxygen as an electron acceptor, but under anoxic conditions they oxidize reduced sulfur (S^{2-}, $S_2O_3^{2-}$, S^0) to sulfate while reducing nitrate. The equation of sulfur denitrification when elemental sulfur (S^0) is used as an electron donor is shown in Figure 1. In addition to nitrate, *T. denitrificans* can also use nitrite, NO or N_2O as an electron acceptor [13, 14].

Practical use of sulfur denitrification is mainly confined to nitrate removal at off-site plants [15-20]. In addition to these, recently, some on-site applications of sulfur denitrification have been suggested. Zhang & Flere[19] presented the in situ remediation of nitrate-nitrogen by using the pond-system. Yabe *et al.*[21] tested the removal of nitrate-nitrogen discharged from a farm by the addition of particles made of sulfur and limestone into a drainage canal. At present, these on-site applications are limited. As regards to gas emissions, Koenig & Liu[17] reported that N_2O production was under 0.8% of the nitrate-nitrogen fed into the sulfur packed column. But there are very few works concerning N_2O or NO production during sulfur denitrification.

In this study, column and batch experiments were conducted to demonstrate the efficiency of addition of sulfur into soil as an electron donor for denitrification . Subsequently, the ability of nitrate removal and the gas emissions during sulfur denitrification were investigated.

Table 1
Sulfur denitrifying bacteria

name	characteristics
Thiobacillus denitrificans	obligately autotrophic colorless sulfur bacteria
Thiomicrospira denitrificans	obligately autotrophic colorless sulfur bacteria
Thiosphaera pantotropha	facultatively autotrophic sulfur bacteria, aerobic denitrifier
Beggiatoa alba	filamentous bacteria

$$\underset{\text{electron donor}}{5S^0} + \underset{\text{electron acceptor}}{6NO_3^-} + 2H_2O \rightarrow 5SO_4^{2-} + 4H^+ + 3N_2\uparrow$$

Figure 1. Sulfur denitrification

2. MATERIALS AND METHODS

2.1. Collection and analyses of the soil

The soils used in the experiments were collected from a paddy field at the Saitama Agricultural Station in Japan. The soil (I) was submerged in a KNO_3 solution (about 20 mgN/L) for two months after collection. The soils were dried and sieved by a 5mm mesh before the characteristics were measured. Pure water (100mL) was added to 10 g of sieved soil and the mixture was sonicated at 40W for 10 minutes. The slurry was filtered through a disposable filter (DISMIC, pore size = 0.45 μm, Advantec) and chemical indices were analyzed in the filtrate. The true and apparent specific gravities were measured by a 100mL picnometer and 50mL measuring cylinder, respectively. The gravimetric water content was analyzed by the infrared heating water meter (FD-600, Kett). The three phases of the soil were determined by these measured physical indices (Table 2).

Table 2
Soil characteristics

indices			soil No. →	(I)	(II)
chemical	NO_3^- (mgN/g dry soil)			0.037	0.046
	NO_2^- (mgN/g dry soil)			0.000	0.000
	SO_4^{2-} (mgS/g dry soil)			0.055	0.019
	DOC (mgC/g dry soil)			0.448	0.208
	pH			6.30	
physical	gravimetric ratio	solid content		98.0 %	
		water content		2.0 %	
	volumetric ratio	solid content		40.1 %	
		water content		2.2 %	
		air-filled porosity		57.7 %	
	ratio in pore space	air-filled		96.4 %	
		water-filled		3.6 %	

blank : not measured

2.2 Column experiment (1)

2.2.1 Column set-up

The soil-columns made of vinyl chloride were set up as shown in Figure 2. The soil characteristics are as given in Table 2 under soil No.(I). Column A was packed with only soil and operated as a control, while Column B was packed with a mixture of soil and elemental sulfur powder sufficient to reduce 525 g of nitrate-nitrogen. A substrate containing only nitrate-nitrogen and no organic compounds was diluted with tap water and fed into the top of the columns at the constant flow rate of 90 mL/h. Soil-pore water was collected from the soil-pore water collection kit (Daiki, Japan) laid at each sampling point (No.1 - 5). Inflow and outflow water were also collected. Gas sample was taken from the gas phase over the soil surface. The top of the column was sealed during the initial 47 days, but it was opened to the atmosphere after 47 days because too reduced condition caused the sulfate reduction.

2.2.2 Analyses

NO_3^-, NO_2^-, SO_4^{2-}, TOC, and pH were measured in the samples filtered by the disposable filter (DISMIC, pore size = 0.45 μm, Advantec). NO_3^-, NO_2^-, and SO_4^{2-} were analyzed by Capillary Ion Analyzer (CIA, Waters). TOC and pH were determined by TOC analyzer (TOC-500, Shimadzu) and the electrode (TOA), respectively.

N_2O concentration in the gas sample was determined by gas chromatography (GC) equipped with electron capture detector (ECD). The analytical condition of GC-ECD (GC-8AIE, Shimadzu) is shown in Table 3. The concentration of dissolved N_2O was measured by the headspace method. Fixed quantity (20 - 40 mL) of water sample was added gently into a 50 mL glass vial without incurring of the release of N_2O from the water phase, and fixed quantity (1 - 3 mL) of Hibitane (5 w/v% gluconate solution; Sumitomo Pharmacy) was subsequently added for the inactivation of bacterial function. Then the vial was sealed with a butyl-rubber septum together with an aluminum seal and shaken. After this, the vial was kept intact at 20°C for 1 hour. The gas phase (0.5 mL) was taken and injected to GC-ECD using a gas-tight syringe (1mL; Hamilton) for the analysis of N_2O. The concentration of N_2O was determined by comparing the chromatogram area with the areas of the standards.

2.3. Batch experiment

2.3.1 Settings

To examine the efficiency of sulfur as an electron donor for denitrification, a series of batch experiments using various electron donor, such as S^0, FeS, glucose, or cellulose, was carried out. The set conditions are as shown in Table 4. $CaCO_3$ was added for pH adjustment except for case (f). Dry paddy soil (No. (I)),

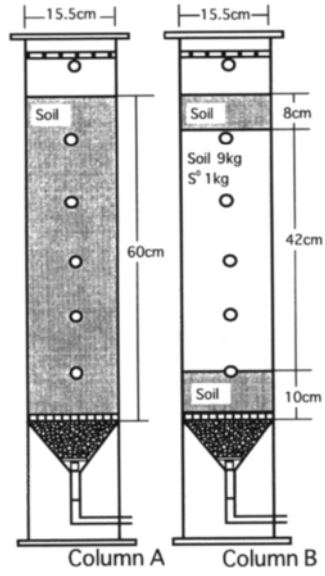

Figure 2. Soil columns
A: control, B: sulfur addition

Table 3
Analytical condition of GC

GC	GC-8AIE (Shimadzu)
column	glass column (2.6mm x 3.0mm)
	Polapak-Q (80/100 mesh)
carrier	N_2 (30mL/min.)
temp.	detector 340℃
	column 70℃

Table 4
Batch experiment settings

	added compounds
a	S^0 + $CaCO_3$
b	FeS + $CaCO_3$
c	glucose + $CaCO_3$
d	cellulose + $CaCO_3$
e	$CaCO_3$
f	none

each electron donor, and $CaCO_3$ were mixed at a weight ratio of 8.5 : 1.0 : 0.5. Fifteen grams of the mixture and 10 mL of 100 mgN/L KNO_3 solution were put into a 50 mL vial. The vial was sealed with a butyl-rubber septum and an aluminum seal after the air phase of the vial was purged with N_2 gas for maintaining anoxic conditions. Fifteen vials were prepared for one experimental condition to serve for sampling with time.

2.3.2 Sampling and analyses

The gas sample (0.5 mL) was taken from the gas phase and injected to GC-ECD for N_2O measurement. After the gas sampling, the vial was opened and 25 mL of pure water was added into the vial and the sample was sonicated by the ultrasonic homogenizer at 40W for 10 minutes. The slurry was filtered by the disposable filter (DISMIC, pore size = 0.45μm, Advantec) and NO_2^-, NO_3^-, and SO_4^{2-} were analyzed by CIA.

2.4. Column experiment (2)

2.4.1 Column set-up

To examine the influence of continuous operation of the soil column into which each electron donor (Table 5) was added with $CaCO_3$ in layer form, the columns as shown in Figure 3 were constructed. The packed soil was the paddy soil (II). The substrate contained only KNO_3, which was fed into the column at 90mL/h. The top of the column was sealed throughout this experiment for keeping anoxic condition.

Table 5
Column experiment (2) settings

	added compounds
A	none
B	$CaCO_3$
C	FeS + $CaCO_3$
D	S^0 + $CaCO_3$
E	cellulose + $CaCO_3$
F	S^0 + cellulose + $CaCO_3$

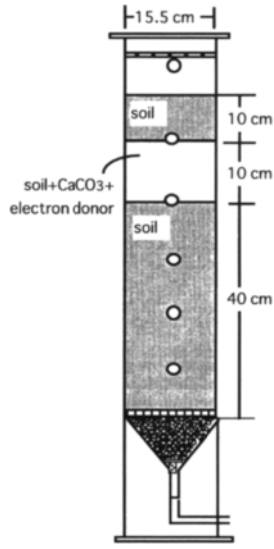

Figure 3. Layered electron donor column

2.4.2 Samplings and analyses

The sampling points were as same as shown in the previous column experiment. In addition to the analyzed compounds given in section 2.2, NO of the gas phase was also measured. For the NO analysis, 0.5 mL of the gas phase was taken and analyzed by NO analyzer (280NOA, SIEVERSE), which detects NO by chemiluminescence. The concentration of NO was calculated by comparing the peak area with the standards.

3. RESULTS AND DISCUSSION

3.1. Column experiment (1)

Figure 4 shows the profiles of dissolved N_2O, NO_3^-, and SO_4^{2-} in the column experiment (1). In Column A, there was little variation of the concentration of NO_3^- and SO_4^{2-} with depth. On the other hand, in Column B, where elemental sulfur was added, NO_3^- concentration decreased with depth, whereas SO_4^{2-} concentration increased. This clearly indicates the occurrence of sulfur denitrification in Column B. The maximum concentrations of dissolved N_2O were detected after about 5 days in both columns. The concentrations increased with depth in both columns on the 5th day, but the concentration of N_2O in Column B was kept low and decreased with depth by the 32nd day. After about 40 days, the concentration of SO_4^{2-} decreased and the soil color became black, especially in the lower part of Column B. This suggests sulfide formation by sulfate reduction after disappearance of NO_3^-. Therefore, the columns were operated with opened top for avoiding the excessive reductive condition. After this change, the sulfate reduction was constrained while denitrification took place, but the ability of NO_3^- reduction was weakened due to inhibition by oxygen from the top. The N_2O accumulated, especially in the top layer of Column B, around day 81.

Nitrate-nitrogen was sufficiently removed by sulfur denitrification in the soil. During the start-up state, small amount of nitrate-nitrogen was removed by the column. This was caused by low activity of nitrate reduction, which resulted in much N_2O accumulation in this period. After that, N_2O reduced in the soil column packed with elemental sulfur with increased denitrification activity. However, pH decline due to H^+ formation with progress of sulfur denitrification and the insufficient reductive condition by opening the top, caused the accumulation of N_2O around day 81.

Figure 5 shows the comparison of NO_3^--N removal and N_2O fluxes from Column B and those values in the actual paddy field, where the groudwater containing high concentration of NO_3^--N (20 - 30 mgN/L) was irrigated with no addition of electron donors for denitrification. The removal of NO_3^--N was determined by multiplying the difference between the average concentration of the inflow and outflow by the flow rate. N_2O flux from the column was calculated by the temporal change of the N_2O concentration in the gas phase.

Increase in nitrate-nitrogen removal and decrease in N_2O emission were recognized with progress of sulfur denitrification in Column B. Nitrate-nitrogen was sufficiently removed and N_2O emission was suppressed especially during the period of increased denitrification activity (14 - 32 days). The nitrate reduction rate at the surveyed field in June, when the paddy field was gradually submerged by irrigation, was relatively high. But the nitrate reduction rate of Column B during the period of increased denitrification activity (14 - 32 days) was much higher than the values at the surveyed paddy field. The N_2O flux from Column B was much lower than the average N_2O flux at the field survey. This indicates that sulfur addition into soil will increase denitrification activity and suppress N_2O emission.

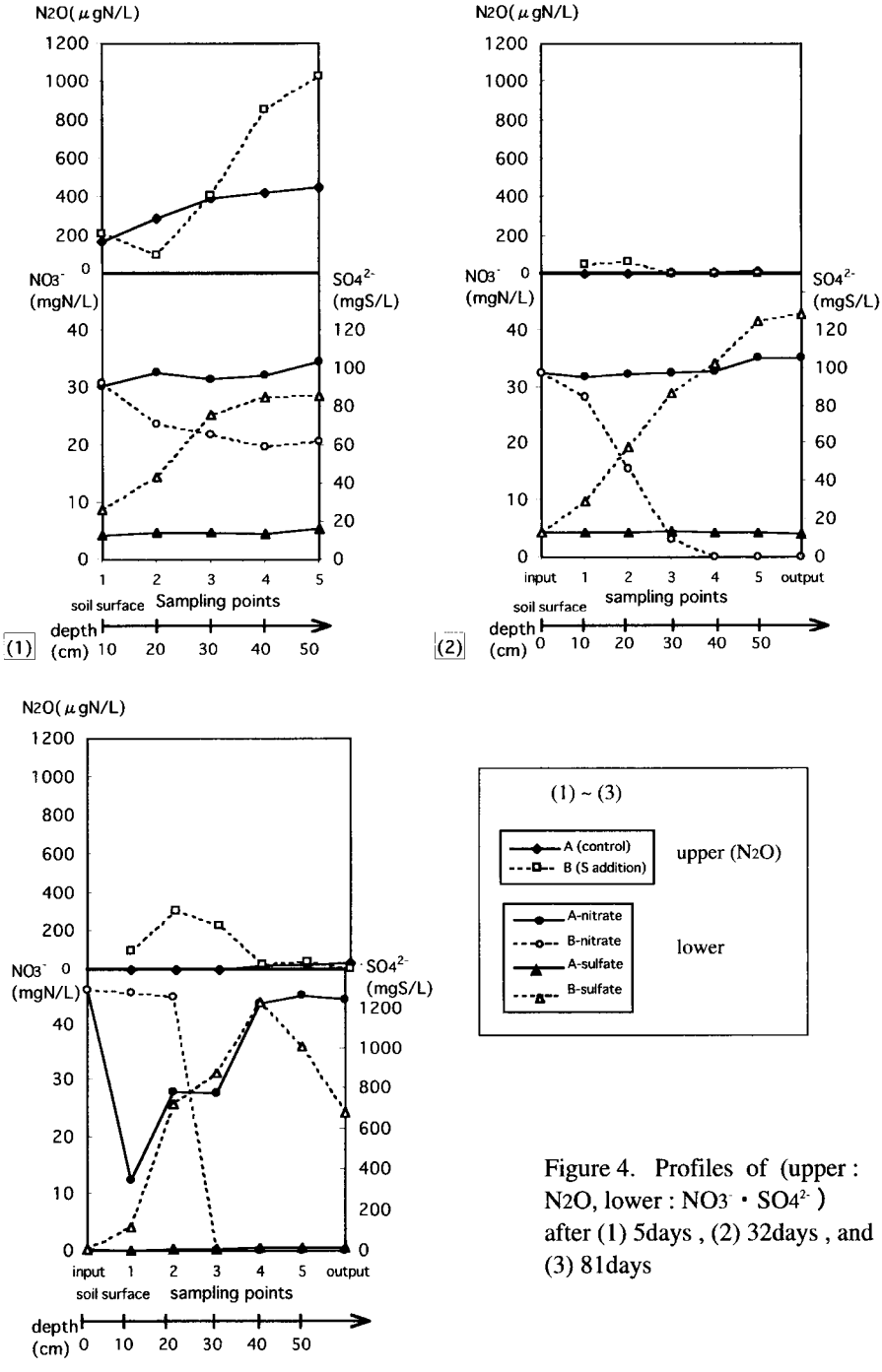

Figure 4. Profiles of (upper : N_2O, lower : NO_3^- · SO_4^{2-}) after (1) 5days , (2) 32days , and (3) 81days

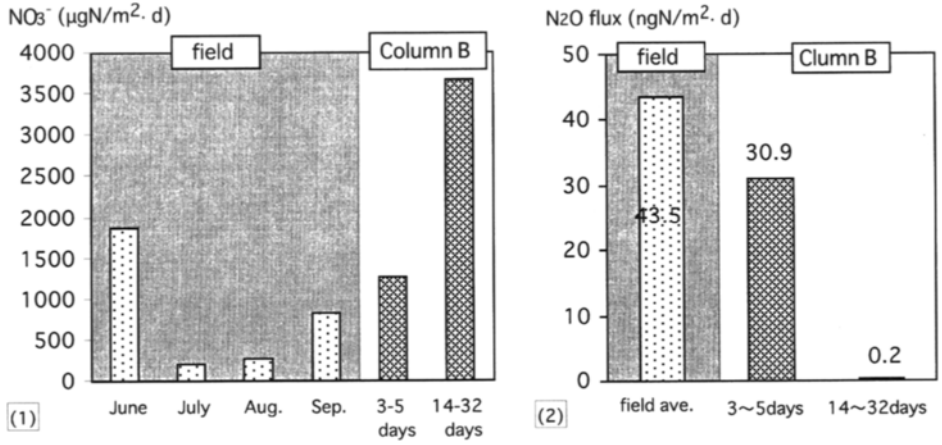

Figure 5. Comparison of the nitrate reduction rate (1) and N_2O flux (2) from the Column B (S^0 addition) with the values in the surveyed paddy field.

3.2. Batch experiment

Figure 6 shows the temporal changes of N_2O in the gas phase of the vial supplemented with different electron donors. When sulfur was added (a and b), the concentration of N_2O did not exceed 60 ppm. On the other hand, in the other cases (c - f), the concentration of N_2O reached about 6000 ppm after a few days. When $CaCO_3$ was added together with each electron donor, the produced N_2O decreased in a few days. However, it took a long time for N_2O reduction when there was no electron donor (f).

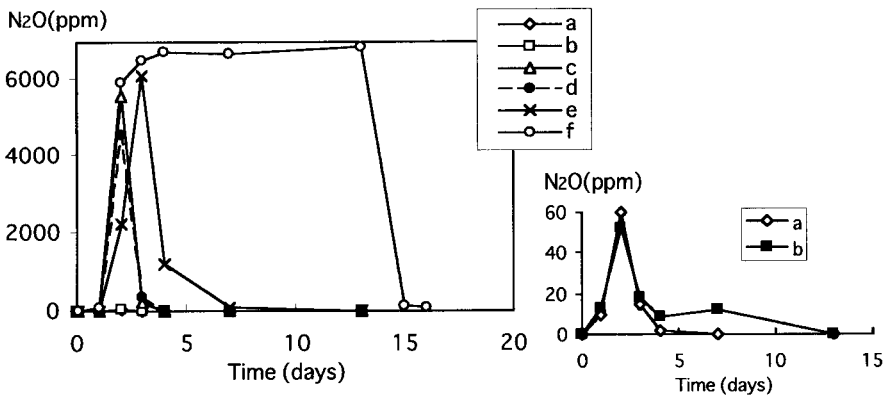

Figure 6. Temporal change of N_2O concentration under each electron donor. (a: S^0+$CaCO_3$, b: FeS+$CaCO_3$, c:glucose+$CaCO_3$, d:cellulose+$CaCO_3$, e:$CaCO_3$, f:none, The right figure shows magnified results of (a) and (b).)

Figure 7 shows the NO_3^--N reduction rate and the ratio of the N_2O production rate to the NO_3^--N reduction rate (term "N_2O emission ratio" hereafter). The NO_3^--N reduction rate in the case of $CaCO_3$ addition (e) was 1.7 times higher than control (f). In the cases where organic electron donors were used, comparatively higher rates of 2.7 (c) and 2.1 (d) times were obtained as against $CaCO_3$ alone (e). When sulfur was used, little increase in the reduction rate was observed. The N_2O emission ratio was highest under no electron donor (f) , while it was much suppressed in the cases of sulfur addition (a and b).

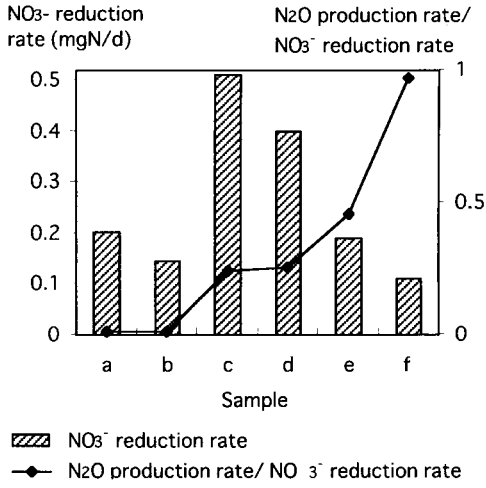

Figure 7. NO_3^--N reduction rate (column) and the ratio of the N_2O production rate to NO_3^--N reduction rate (N_2O emission ratio, line)

(a: S^0+$CaCO_3$, b: FeS+$CaCO_3$, c:glucose+$CaCO_3$, d:cellulose+$CaCO_3$, e:$CaCO_3$, f:none)

3.3. Column experiment (2)

Temporal changes of N_2O and NO in the gas phase of the column experiment (2) are shown in Figure 8. NO was emitted after a few days and decreased immediately under all the conditions. The concentration of NO in the FeS column (C) was much higher than NO in the other columns. In Column F, the concentration of NO increased again after 43 days. The concentrations of N_2O were much higher than those of NO. A high concentration of N_2O was detected especially in Column F, where both cellulose and S^0 were added. N_2O decreased and reached a few ppm after 34 days in Columns A, B, D, E, and F. But the accumulation of N_2O continued throughout this experiment in Column C, where FeS was added as an electron donor. In Columns E and F, the concentration of N_2O increased again after 47 days.

Figure 9 shows the NO_3^--N profile of each column after 41 days. The decline of the concentration of NO_3^--N was scarcely detected in Columns A, B, and C. In Columns D, E, and F, the reduction of NO_3^--N occurred especially in the zone where electron donor was added. The concentration of NO_3^--N at the sampling point No.2 was almost zero in these columns (D, E, and F). After 40 days, the formation of sulfide was observed as black color especially in the columns where cellulose was added. The reappearance of NO in Column F and N_2O in Columns E and F as mentioned above can be attributed to the inhibition of NO and N_2O reduction by this sulfide. In addition to sulfide formation, the pH decline as sulfur denitrification progressed was also a serious problem. But the addition of $CaCO_3$ in this experiment worked effectively in buffering pH.

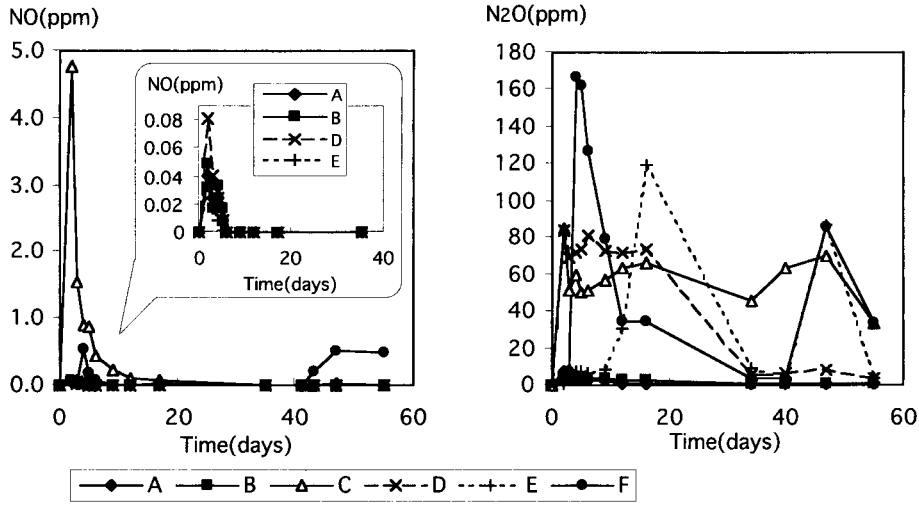

Figure 8. Temporal changes of NO(left) and N_2O(right) with different electron donors. (A: none, B: $CaCO_3$, C: FeS + $CaCO_3$, D: S^0 + $CaCO_3$, E: cellulose + $CaCO_3$, F: S^0 + cellulose + $CaCO_3$)

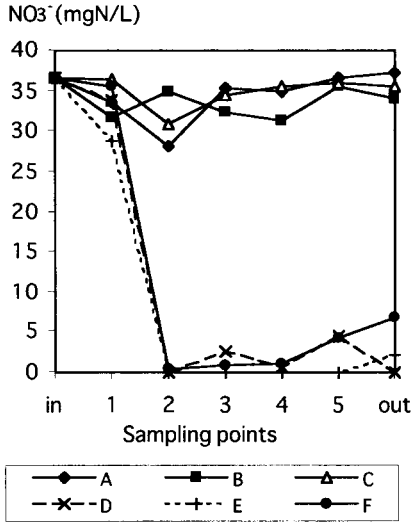

Figure 9. Profiles of NO_3^- after 41 days (A: none, B: $CaCO_3$, C: FeS + $CaCO_3$, D: S^0 + $CaCO_3$, E: cellulose + $CaCO_3$, F: S^0 + cellulose + $CaCO_3$)

4. CONCLUSIONS

The addition of sulfur into the soil promoted denitrification and decreased the N_2O emission ratio as compared to the values from the actual paddy field. Especially in the case when inorganic sulfur was added to the soil with $CaCO_3$, the N_2O emission ratio was kept extremely low.

The use of organic material was more effective in obtaining high reduction rates of nitrate-nitrogen. But the organic material promoted the occurrence of sulfate reduction and formation of sulfide. Sulfide caused inhibition of NO and N_2O reduction resulting in accumulation of these compounds. Therefore, it is concluded that elemental sulfur is a more appropriate additive to the soil-water system as an electron donor for denitrification than organic materials and FeS.

The NO emission was negligible because of its much lower concentration than N_2O. The column experiment demonstrated the occurrence of sufficient nitrate removal even if sulfur was added into the soil in the layer form, which is more applicable form than mixing.

The results of this study demonstrated the effectiveness of adding sulfur into soil for in-situ removal of nitrate-nitrogen and elimination of N_2O emission, but further study is required for practical use of sulfur in soil-water environments

REFERENCES

1. IPCC, 1996. Climate Change 1995: The Science of Climate Change. Contribution of Working Group I to the Second Assessment Report of the Intergovernmental Panel on Climate Change.J.T.Houghton, L.G.Meira Filho, B.A.Callander, N.Harris, A.Kattenberg and K.Maskell (eds.), Cambridge Univ. Press, Cambridge and New York.
2. N. Arai , A. Matsunami, K. Matsumoto, K. Kitagawa, N. Kobayashi and K. asai, 1997. Anal. Com., 34, 205-206.
3. K.M. Hiscock, J.W. Lloyd and D.N. Lerner, 1991. Wat. Res. , 25 (9), 1099-1111.
4. B. Singh, Y. Singh and G.S.Sekhon, 1995. J. Contami. Hydro., 20, 167-184.
5. W. L. Zhang, Z. X. Tian, N. Zhang and X. Q. Li, 1996. Agriculture, Ecosystems and Environment, 59, 223-231.
6. G. D.Agrawal, S.K. Lunkad, T. Malkhed, 1999. Wat. Sci. Tech. , 39 (3),67-75.
7. J.I.Prosser, 1989. Adv. Microbial Physiol., 30, 125-181.
8. R.Knowles, 1982. Microbial.Rev., 43-70.
9. H.Kawashima, M.J.Bazin, and J.M.Lynch , 1996. Ecological Model., 87, 51-57.
10. K.Hasegawa, K. Hanaki, T. Matsuo and S. Hidaka, 1998. J. Japan Soc. Wat. Environ., 21(10), 676-682. in Japanese
11. J. G. Kuenen, 1989. J. T. Staley , M. P. Bryant, N. Pfennig and J. G. Holt (eds.), Bergy's manual of systematic bacteriology volume 3, pp1834-1871.
12. J. G. Kuenen, L.A. Robertson, and O.H. Tuovinen , 1992. In: A. Balows, H. G. Truper, M. Dworkin, W. Harder and K. H. Schleifer (eds.), The Prokaryotes Second Edition, Springer-Verlag, New York, pp2638-2657

13. K. H. Lee and K. L. Sublette, 1990. Applied Biochem. Biotech., 24/25, 441-445.
14. K.Baalsrud and K. S. Baalsrud, 1954. Arch. Mikrobiol., 20, 34-62.
15. S. Hashimoto, K. Furukawa, and M. Shioyama, 1987. J. Ferment. Technol., 65(6), 683-692.
16. H. Furumai, H. Tagui and K. Fujita, 1996. Wat. Sci. Tech., 34(1-2), 355-362.
17. A. Koenig and L.H. Liu, 1997. Proc. IAWQ 8th International Conf. on Anaerobic Digestion, 1, 299-306.
18. Z.Wang, , 1998. Wat. Sci. Tech., 37(9), 97-104.
19. T.C. Zhang and J. M. Flere, 1998. Wat. Sci. Tech., 38(1), 15-22.
20. Y. Xiushan, G. Garuti and A. Tilche, 1993. Biotech. Let., 15(5), 531-536.
21. T.Yabe, T. Nishimura, H. Masujima and A. Yatagai, 1999. Proc. 33th Japan Soc. Wat. Environ., 256. in Japanese

FISHing for biomass in activated sludge mixed liquor: the slippery VSS fraction

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Among the issues confronting activated sludge researchers are the assessment of the active biomass mediating the process and the delineation of the key microbial groups comprising the total population. More specific to biological excess phosphate removal (BEPR) is the quantification of that portion of the population responsible for the release and subsequent uptake of soluble phosphate-the polyphosphate accumulating organisms (PAO's). This study attempts to address these questions by investigating the active microbial community within pilot- and full-scale BEPR systems. Microbial community analysis was conducted on the full-scale plant using fluorescent *in situ* hybridization (FISH) and dot blots. The alpha and beta Proteobacteria were found to dominate this activated sludge population. Enrichment procedures performed on a continuous pilot-scale plant designed to enhance the growth of PAO's, followed by probing with fluorescently labeled oligonucleotides, also showed the alpha and beta Proteobacteria to be dominant. This study shows how the application of FISH can be used for determining that fraction of the measured volatile suspended solids (VSS) present as total active biomass (X_a) and PAO's (X_{PAO}).

1. INTRODUCTION

Engineers have traditionally used mixed liquor volatile suspended solids (MLVSS) as a 'lumped' indication of the active biomass within activated sludge systems. Although the MLVSS parameter has the advantage of fitting directly into mass balance equations, it has the disadvantage of not providing a true indication of the active biomass present. This parameter represents not only X_a but also endogenous residue (dead cellular material; X_e) and inert particulate COD (originating from the influent; X_{ii}) present in the sludge [1].

Within Activated Sludge Model No.2 [2], X_a of nitrification/denitrification biological excess phosphorus removal (NDBEPR) sludge encompasses the following components;

- nitrifying organisms (X_{AUT}), responsible for nitrification;
- the 'all-rounder' heterotrophic organisms (X_H), responsible for fermentation in the anaerobic zone, denitrification in the anoxic zone, and chemoheterotrophic activity in the aerobic zone;
- X_{PAO}, responsible for the accumulation of polyphosphate in the aerobic zone.

However, efforts to calibrate and validate the model kinetic and stoichiometric parameters do not currently involve the direct, experimental determination of mixed liquor concentrations of these microbial populations. Through advances in molecular biology, in particular, developments in the field of microbial evolution [3], tools have become available which may prove useful in furthering the descriptive and predictive capacity of current mathematical models for BEPR.

Direct techniques are now the method of preference for quantifying bacteria in a variety of ecosystems [4,5]. For the activated sludge process in particular such techniques have been found to be more representative of the microbial community compared to indirect, cultivation dependant methods [6]. The FISH approach targets ribosomal ribonucleic acid (rRNA) within intact bacterial cells using oligonucleotides that are complementary to conserved signature sequences of phylogenetically defined taxa. Such probes have been designed for the following taxonomic levels;

- domains *Archaea*, *Bacteria* and *Eukarya* [7];
- intermediate family levels; viz.,alpha, beta, gamma subclasses of the family Proteobacteria [8]; and the Actinobacteria [9]; and,
- lower genus levels i.e., *Acinetobacter* [10].

Since probe penetration through the cell wall can sometimes be problematic (e.g., Gram positive bacteria having thick cell walls) [11], dot bot hybridizations can be applied to verify the community profile obtained by *in situ* methods. Here, total nucleic acid is extracted from the sample of interest and immobilised on a solid support medium followed by probing with oligonucleotides of interest. Such an approach was first used to monitor population shifts in the rumen of cattle [4].

Research undertaken to determine the PAO population implicated in BEPR identifies particular bacterial phylogenetic families as being predominant in either SBR's operated in aerobic/anaerobic cyclic conditions or continuous anaerobic/anoxic/aerobic conditions to stimulate BEPR. These studies indicate the predominance of the beta Proteobacteria and Actinobacteria in BEPR [12,13]. Sudiana *et al.*, (1998)[14] found that the beta Proteobacteria group predominated in activated sludge communities which had been acclimatised with acetate as the major carbon source under phosphorus limited and rich conditions.

The use of DAPI for detecting polyphosphate in BEPR activated sludges was first reported by Streichan *et al.*, (1990)[15]. DAPI is routinely used as a DNA intercalating fluorescent dye for the *in situ* detection of single cells; the DNA-DAPI complex emits a blue-white colour. For polyphosphate detection the DAPI-polyphosphate complex fluoresces bright yellow.

This study utilises the popular approach of fluorescent *in situ* and membrane hybridizations with oligonucleotide probes in order to investigate the phylogenetic affiliation of the predominating organisms of both full- and pilot-scale BEPR processes. Probe results for the determination of X_a and X_{PAO} are converted to mass fractions of measured system VSS under defined operational conditions.

2. METHODS AND MATERIALS

2.1. Full-scale process description

The activated sludge process at Darvill Wastewater Works (WWW) (KwaZulu-Natal, South Africa) consists of conventional primary treatment and sedimentation, followed by secondary biological treatment. The activated sludge process has been designed to conform closely to the Johannesburg process configuration. The activated sludge plant consists of three aeration basins (total volume = 19 600 m^3) with five surface aerators (75 kW) in each basin. Refer to Figure1 for a diagrammatic description of the Darvill WWW activated sludge plant. Settled sewage flow is fed directly to the anaerobic zone together with a volatile fatty acid (VFA) containing stream originating from the supernatant of primary sludge fermenters. Denitrification of the return sludge is encouraged by feeding a minor fraction (*ca.* 4%) of the settled sewage into the pre-anoxic zone. A description of settled sewage composition data flowing into the activated sludge plant over a two year period (January 1995 to February 1997) is given in Table 1.

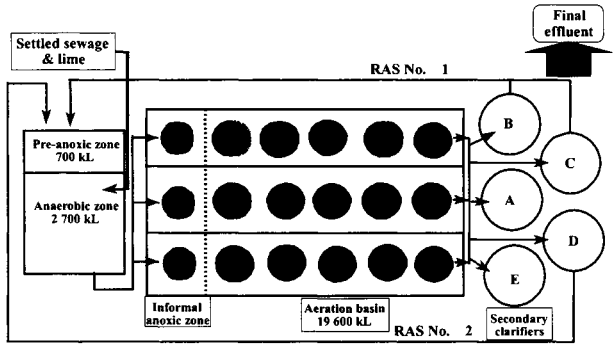

Figure 1 Schematic representation of Darvill activated sludge plant modified for bio-P removal.

Table 1
Settled sewage composition to Darvill activated sludge plant (average for a two year period; January 1995 to February 1997)

Determinant	Mean
COD	242.9 mg/L
TKN	24.8 mgN/L
Ortho-P	5.6 mgP/L
TP	8.5 mgP/L

2.2 Pilot Scale Unit configuration and layout

The pilot plant was designed and modeled upon the 3-stage Phoredox process and operated at 20°C (±1°C). A schematic design of the pilot plant is given in Fig. 1. Reactor configuration consisted of the following zones: anaerobic (AN; 8 L); anoxic (AX; 4 L); first aerobic (AE1; 10 L); and second aerobic (AE2; 10 L). The clarifier (2.5 L), situated downstream of the reactors, was positioned at a 60° angle to the horizontal. Target influent flow rate (Q_i) was set at 36 L/d; settled sewage was fed directly to the AN zone using a peristaltic pump (Gilson). The s-recycle, pumped (Gilson) from the clarifier to the AN zone, was set at a ratio of 1:1, with respect to Q_i. The a-recycle, from AE2 to the AX zone, was pumped (Watson-Marlow) at a ratio of 3:1, with respect to Q_i. System sludge age (R_s) was maintained at 10 d for the duration of experimentation. Oxygen uptake rate (OUR) was measured in AE2 by means of a dissolved oxygen (DO) probe and meter (Randall *et al.*, 1991). Air sparged into the aerated zones was controlled such that the aquarium air pumps switched the supply on and off in accordance with the lower and upper set DO points of 2.0 and 5.0 mgO/L, respectively.

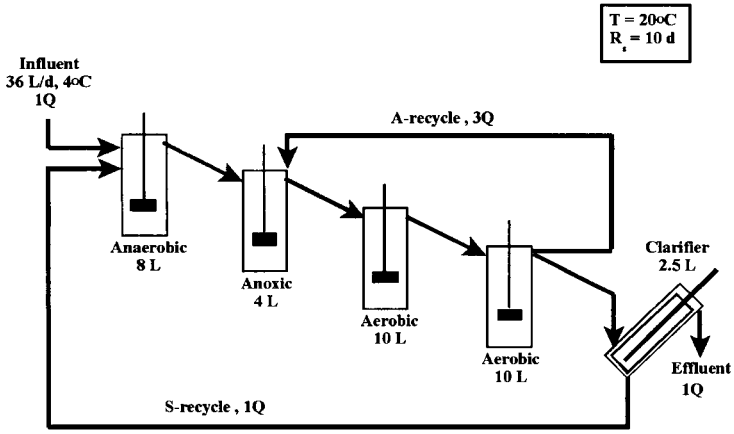

Figure 2 Schematic representation of pilot-plant

2.3. Sampling and cell fixation

Grab samples of activated sludge mixed liquor were collected from the anaerobic, anoxic, and aerobic zones of the full scale process, and from the pilot scale EBPR process at the end of the experimental period. Samples were fixed for 2 hours at 4°C with 3% paraformaldehyde/PBS by the addition of three volumes of fixative to one volume of sample. Cells were then washed in 1 x PBS (130mM NaCl, 10mM Sodium phosphate buffer, pH 7.2) and resuspended in PBS/cold absolute ethanol (1:1 v/v). For fixation of Gram positive cells activated sludge was added to ethanol to a final concentration of 50% (v/v).

2.4. Membrane Filtration and staining with DAPI

Membrane filtration was carried out as described by Porter and Feig (1980)[16]. Dual staining of cells with DAPI and fluorescent oligonucleotides was modified from the method of Hicks *et al.* (1992)[17] so that cells were stained after *in situ* hybridization with DAPI (0.33μg/mL). for 5 min. For detection of PAO's cells were stained with 50 μg/mL DAPI for 20 seconds.

2.5. Oligonucleotide probes

The following oligonucleotide probes were used: probes specific for the alpha, beta, and gamma subclass of Proteobacteria (ALF1b, BET42a and GAM42a, respectively, Manz *et al.*, 1992)[8]; a probe specific for Actinobacteria (HGC69a, Roller *et al.*, 1994) a probe specific for all hitherto sequenced bacteria (EUB338, [7]). Probes were synthesized and labeled with either rhodamine (red) or fluorescein (green) by Roche Molecular Biochemicals, Germany.

2.6. FISH hybridization

Aliquots of 5μL fixed sludge were applied to poly-lysinated slides and allowed to air dry. Spotted cells were then dehydrated by serial immersion through 60%, 80% and 96% (v/v) ethanol (3 min each). Samples of 10μL hybridization solution (0.9M NaCl, 20mM Tris/HCl, pH 7.2, 0.01% SDS, 50 ng probe, X % (v/v) formamide) were applied to each spot and and incubated for two hours at 46^{0}C in an isotonically equilibrated humidity chamber. Formamide concentrations (v/v) for each probe was as follows; alpha, 25%; beta 35%; gamma,35%; HGC25%. Probe was removed from the slide by rinsing with 2mL prewarmed washing solution (20 mM Tris/HCl, 0.01% SDS, 5mM EDTA, Y M NaCl; the salt concentration, Y, was adjusted to the formamide concentration, X , in the hybridization buffer according to the formula of Lathe,1985 [18]. Slides were rapidly transferred into washing solution and incubated at 48^{0}C for 20 min. Slides were then rinsed briefly with distilled water, air-dried and mounted in vectashield antifading solution for viewing by microscopy.

2.7. Microscopy and Image Analysis

Cells were visualised with a Zeiss Axiolab microscope fitted for epifluorescence with a 50W mercury high- pressure bulb and Zeiss filter sets 02, 09 and 15. Images were captured using a Sony (Germany) CCD camera. Image analysis was carried out using the Zeiss KS 300 imaging system.

2.8. Nucleic acid extraction and membrane hybridization

Total nucleic acids were extracted from about 0.2g of activated sludge (wet weight)[3]. Membrane hybridizations were performed as described by Manz *et.al.* (1992)[8].

3. RESULTS AND DATA INTERPRETATION

The microbial community represented in Table 2 was analysed by measuring the area of cells that displayed probe conferred fluorescence and expressing this as a percentage of the area that bound DAPI. The results indicated in Table 2 represent the biomass within the aerobic zone and the community composition was found to be stable throughout the system. Table 2 includes results obtained from dot blot hybridizations performed for the full-scale plant. For dot blots, group-specific probes are represented as percentages of probe EUB338. The main motivation behind performing the dot blots was to verify the community profile obtained from the FISH experiments. Overall, the dot blot hybridizations showed the same profile as that obtained by FISH and indicated that the alpha-Proteobacteria predominated the sludge in comparable numbers to the beta-Proteobacteria. This trend in community profile was maintained even after the stringency for the alpha-Pproteobacteria probe was increased by the addition of up to 30% (v/v) formamide in the hybridization buffer.

TABLE 2
Probe specific counts (*in situ*) expressed as a percentage of total cells stained with DAPI.

	Toti non-BEPR (%)	Enriched BEPR Pilot-plant (%)	Full-scale Nutrient Removal (%)	
			FISH	Dot Blot
α	11	19	20	30
β	25	23	30	37
γ	14	17	13	14
HGC	13	11	8	12
Total	61	69	79	96
EUB338	73	78	80	100
PAO*	n/d	55	25	n/d

*as revealed by DAPI staining

Table 3 shows the percentage contribution of endogenous residue to the VSS for the pilot-scale plant. This was determined by comparing the number of cells displaying DAPI conferred fluorescence with the total number of cells visible by light microscopy. Since DAPI intercalates the bacterial chromosomal DNA, those cells not showing DAPI fluorescence were assumed to have their DNA degraded and therefore constitute endogenous residue, X_e. VSS readings for normal activated sludge systems also contain an inert fraction (X_{ii}) and an endogenous residue fraction X_e in addition to the active biomass (X_a). In order to experimentally determine that fraction of the VSS that exists as active bacterial biomass, the percentage X_e as determined in Table 3 must be subtracted from the measured VSS reading. Since, at the time of sampling, the pilot-scale BEPR system was fed with a single readily biodegradable substrate source (acetate), the X_{ii} fraction for this system can be assumed to be zero. The fraction of the active biomass as VSS can then be determined (see equation 1, results shown in Table 4). By multiplying the EUB/DAPI ratio by the total cell count (DAPI, membrane filtration) (from Table 3) the metabolically active biomass of the sludge in terms of cell numbers can be estimated from eq2 (results shown in Table 4):

VSS as biomass (g/L) = (100-%X_e) × measured VSS (eq 1)

X_a (cells/mL) = (%EUB/DAPI) × DAPI(cells/mL) (eq 2)

From eq 1 and eq 2, the mean VSS per cell for the pilot-scale system can be determined by:

Mean VSS per cell (g/cell) = VSS as biomass (g/L) / X_a (cells/L) (eq 3)

By determining the mean VSS per bacterial cell using the pilot-scale system, the VSS as biomass for the full-scale BEPR system can be calculated by extrapolation from equation 4 (results shown in Table 4):

VSS as biomass (g/L)= Mean VSS/cell (g) × X_a (cells/L) (eq 4)

Table 3
Percentage endogenous residue (X_e) for enriched pilot scale plant

	Average cell no./field
(Light microscopy)	410 / 53
DAPI count	226 / 44
X_e(%)	45

Table 4
Biomass expressed in terms of key process functions for nutrient removal (pilot- and full-scale) and non-nutrient removal (full-scale) sludges.

	Full scale Non BEPR (20d sludge age)	Pilot-scale Enriched BEPR (10d sludge age)	Full-scale BEPR (8d sludge age)
S_{bi}(CODmg/L)	800	492	200
Measured VSS (g/L)	3	1.29	2.75
P removal (mg/L)	n/d	40	5.3
DAPI (cells/mL)	3×10^{10}	1.6×10^{10}	8×10^{9}
X_a(eub/dapi) (%)	73	78	80
X_a (cells/mL)	2.19×10^{10}	1.25×10^{10}	6.4×10^{9}
X_e (%)	n/d	45	n/d
VSS as biomass (g/L)	n/d	0.71	0.36
Measured VSS as X_a (%)	n/d	55	13
Mean VSS/cell (g)	n/d	5.68×10^{-14}	n/d
X_{PAO}*(%)	n/d	55	35
X_{PAO}* (cells/mL)	n/d	6.88×10^{9}	2.24×10^{9}
VSS as PAO's (g/L)	n/d	0.39	0.13
X_{PAO}/mgP removed	n/d	1.7×10^{11}	4.2×10^{11}

*as revealed by staining in excess with DAPI

4. DISCUSSION

DAPI staining for the detection of PAO's *in situ* (Figure 3) showed that the PAO population in the enriched culture was much higher than for the full-scale process (Table 2). It can be hypothesised that no fermentative heterotrophs were competing for COD (as acetate) in the anaerobic zone of the enriched culture and a larger proportion of the biomass was dedicated to phosphate release and subsequent uptake in the aerobic zone. In addition, the influent biodegradable COD fraction for the enriched culture is larger by a factor of approximately 2.5 (Table 4).

The VSS fraction present as biomass for the full-scale plant was calculated to be only 13% of the measured VSS value (Table 4). This implies that 87% of the measured VSS for the full-scale system is present as inert material and endogenous residue that has become enmeshed in the sludge mass. It should also be noted that the contribution of the extracellular polymers (secreted by the sludge biomass) to the VSS has not been taken into account.

Equations 1-4 can also be applied for determining the contribution of X_{PAO} to the measured VSS readings for both full- and pilot-scale systems. The results given in Table 4 show the

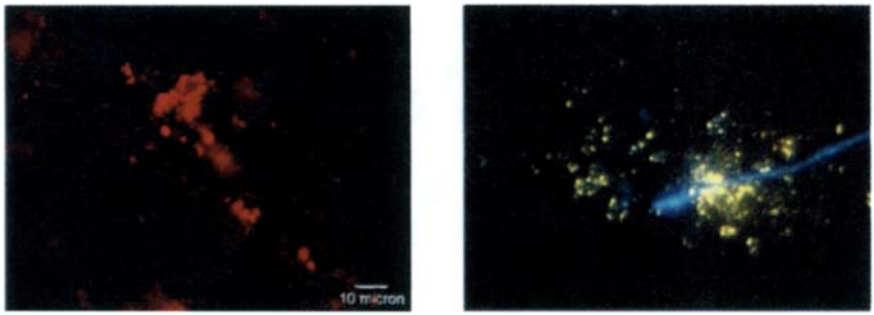

Figure 3. *In situ* hybridization of rhodamine (red) labeled probe Alf1b (left panel) and staining with DAPI for visualisation of PAO's as yellow cells(right panel) for the BEPR pilot-scale system. 10 micron bar applies for both images.

number of PAO's required to accumulate 1 milligram of phosphorus. The values differ by a factor of approximately 2.

While most studies have indicated the predominance of members of the beta Proteobacteria and/or GPBHGC in BEPR sludges [12,13,19,20] the findings presented here indicate that the alpha Proteobacteria are also implicated in BEPR for both the full-scale and enriched cultures (Table 2). Members of the community belonging to the alpha Proteobacteria were visualised to be of a uniform morphotype, being oval shaped and congregated in compact spherical flocs (Figure1). A clear shift in this population was observed to occur from the non-BEPR sludge (11%) to the enriched EBPR sludge (19%). Members of the alpha Proteobacteria have been implicated by Kawaharasaki *et al.*, (1999)[11] as being capable of storing large amounts of polyphosphate although they were not found to predominate the BEPR sludge.

While the FISH method presents a good indication of cell numbers, dot blots represent a good indication of the total rRNA belonging to the group of interest. The measurement of total rRNA is important since we are interested in the relative levels of metabolic activity for the different groups implicated in BEPR. Based on the assumption that rRNA content is directly proportional to growth rate [21,22] the results presented here (Table 2) using dot blots indicate that the alpha Proteobacteria are indeed metabolically active in BEPR systems.

The BEPR community is clearly dispersed over a wide phylogenetic range of at least four families but our findings suggest that the alpha and beta subclasses of the Proteobacteria predominate both BEPR systems studied. While it has been demonstrated how molecular techniques can be applied for the direct determination of key microbial communities in BEPR systems, future work should focus on the comparison of such data to mathematical model outputs.

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REFERENCES

1. Wentzel M C, Ubisi M F and Ekama G A. 1998. *Wat. Sci. Tech.* 37 (4-5): 79-87.
2. Henze M, Gujer W, Mino T, Matsuo T, Wentzel MC, Marais CvR. 1995. Activated sludge No.2, IAWQ Scientific and Technical Report No.3.
3. Woese CR. 1987. Microbiol. Rev. 51(2):221-271.
4. Stahl D A, Flesher B, Mansfield H R and Montgomery L. 1988. *Appl. Env. Microbiol.* 54, 1079-1084.
5. Gersdorf H, Pelz K and Göbel U B. 1993. *FEMS Immunol. Med. Microb.* 6, 109-114.
6. Wagner M, Amann R, Lemmer H and Schleifer K-H 1993. *Appl. Environ. Microbiol.* 59(5), 1520-1525.
7. Amann R I, Krumholz L and Stahl D A. 1990. *J.Bact.* 172, 762-770.
8. Manz W, Amann R I, Ludwig W, Wagner M and Schleifer K-H. 1992. *System. Appl. Microbiol.* 15, 593-600.
9. Roller C, Wagner M, Amann R, Ludwig W and Schleifer K-H. 1994. *Microbiology* 140, 2849-2858.
10. Wagner M, Erhart R, Manz W, Amann R, Lemmer H, Wedi D, and Schleifer K-H. 1994. Appl. Env. Microbiol. 60 (3):792-800.
11. Kawaharasaki M, Tanaka H, Kanagawa T, and Nakamura K. 1999. *Wat. Res.* 33, 257-265.
12. Bond P L, Keller J and Blackall L L. 1999a. *Wat. Sci. Tech.* 39, 13-20.
13. Bond P L, Erhart R, Wagner M, Keller J and Blackall L L. 1999b. *Appl. Env. Microb.*65, 4077-4084.
14. Sudiana I M, Mino T, Satoh H and and Matsuo T. 1998. *Wat. Sci. Tech.* 17, 139-146.
15. Streichan M., Golecki J R., and Schon G. 1990. *FEMS Microbiology Ecology.* 73, 113-124.
16. Porter K G and Feig Y. 1980 The use of DAPI for identifying and counting aquatic microflora. *Limnol. Oceanogr.* 25, 943-948.
17. Hicks R E, Amann R I and Stahl D A. 1992. *Appl. Env. Microb.* 58, 2158-2163.
18. Lathe R 1985. *J. Mol. Biol.* 183 1-12.
19. Kampfer P, Erhart R, Beimfohr C, Bohringer J, Wagner M and Amann R. 1996. *Microb. Ecol* 32, 101-121.
20. Snaidr J, Amann R, Huber I, Ludwig W and Schleifer K-H. 1997. *Appl.Env.Microbiol.* 63, 2884-2896.
21. Schaechter M O, Maaloe O and Kjeldgaard O. 1958. *J. Gen. Microbiol.* 19, 592-606.
22. Delong E F, Wickham G S and Pace N R. 1989. *Science.* 243:1360-1363.

Identification of predominant microbial populations in a non-phosphate removing anaerobic aerobic bioreactor fed with fermented products

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Microbial community of a non-phosphate removing anaerobic-aerobic activated sludge system fed with fermented products was characterized. Predominant microbial populations were initially identified using 16S rDNA cloning analysis, and 34 different sequences were found among 161 clones screened. These sequences were mostly affiliated with the divisions *Proteobacteria* (57.2 % of total clones) and *Cytophagales* (25.5%). Particularly, the genus *Hydrogenophaga* and its related novel bacterial group in the β-I subdivision (49.7%), and the genus *Flavobacterium* (17.4%) were the predominant ones. The abundance of major phylogenetic groups was further evaluated using fluorescent *in-situ* hybridization (FISH) with group-specific rRNA probes. Like the clone library results, bacteria of the β subdivision of the division *Proteobacteria* was the most dominate one and possibly responsible for the substrate metabolism in the process. FISH analysis further detected bacteria from α- and γ-subdivisions, but not from the divisions *Cytophagales* and actinobacteria.

1. INTRODUCTION

The modern activated sludge processes are known for the ability to remove organic pollutants and nutrients from the wastewater stream. One of the advanced innovations is the enhanced biological phosphorus removal process (EBPR), which was usually configured with anaerobic and aerobic zones in sequence. This engineered configuration has provided an adequate growth environment to enrich microorganisms that could accumulate polyphosphate (PAOs). Empirical studies [1-8] on the metabolic behavior of PAOs have suggested that under the anaerobic stage, PAOs could hydrolyze intracellular polyphosphate to orthophosphate, and derive energy for the uptake and storage of carbon substrate such short chain fatty acids into carbon reserved materials (e.g., polyhydroxyalkanoates [PHAs]). Under the subsequent aerobic condition where external carbon substrate was absent, PAOs could consume the stored PHAs for growth and for complete uptake of soluble phosphate that was released anaerobically or present in the wastewater. Through the repetitive practice of the aforementioned metabolic mechanism, the PAO could outgrow other strictly aerobic heterotrophs and predominate in the process. From the engineering aspect, the phosphorus removal could be achieved by constantly wasting the phosphorus-rich sludge followed by incineration and landfill.

Full-scale EBPR processes were very stable when treating the municipal wastewater [2]. However, deterioration of phosphorus removal efficiency could occur under conditions such

as high rainfall flow into the treatment plant, high organic loading [3], unbalanced nutrient condition [4], excess aeration [5], and high nitrate concentration in anaerobic stage [6]. During the deterioration, the predominance of microorganisms other than PAOs have been observed [7, 8] in the processes. These microorganisms could also take up carbon substrate and accumulate it as PHAs in the anaerobic stage without the hydrolysis of polyphosphate, and were named as "G-bacteria" by Cech and Hartmen [9] due to their predominance in an anaerobic-aerobic sludge reactor fed with glucose. Later studies [8,10] have termed these microorganisms as Glycogen Accumulating Organisms (GAOs), because that the GAOs could degrade glycogen intracellular to obtain energy for the uptake of SCFAs, and could store the substrate as intracellular PHAs in anaerobic stage. In the following aerobic stage, GAOs consumed PHAs both for growth and glycogen accumulation. The metabolism employed by GAOs differed from that of PAOs only in the energy source used for the uptake of substrate under anaerobic conditions [7-8,11].

At present, the microbial diversity of GAOs was not fully characterized yet. This was attributed to the experimental technique previously used and the complexity of the microbial diversity in GAOs under different operational conditions. The former difficulty was most often related to the cultivation methods, and could be improved by using ribosomal RNA-based molecular techniques to analyze the microbial diversity (i.e., structure and composition) and spatial distribution [12-20]. In contrast, the complexity of the microbial diversity under different operational conditions has not been closely examined. It was apparent that substrate type (e.g., acetate and glucose) and phosphorus loading would selectively enrich different populations in activated sludge processes [7,21]. In this study, the molecular technique was applied to characterize the microbial community of a non-phosphate removing anaerobic-aerobic bioreactor fed with fermented products.

2. MATERIALS AND METHODS

2.1. Sludge sample

The sludge was sampled from an anaerobic-aerobic sequence batch reactor (A/O SBR) fed with a mixture of fermented substrate of acetate, yeast extract, peptone, magnesium, potassium, calcium [22]. The total volume, hydraulic retention time, and sludge retention time was 8.5 liters, 2days and 10 days, respectively. The influent DOC and PO_4-P are 140 mg C/L and 10 mg P/L. During more than one year of cultivation, the total suspended solids (TSS) and sludge phosphorus content (% of TSS) were fluctuated around about 4800 mg/L and 2.5%, respectively. During the anaerobic stage, the majority of the DOC fed was taken up with no release of orthophosphate.

2.2. DNA Extraction and 16S rDNA clone library construction

The benzyl chloride method [23] was used to extract the sludge DNA. For PCR amplification of 16S rDNA from extracted DNA, 10 μl of 2mM dNTP, 10μl of 10X PCR buffer, 5μl of 50mM magnesium, 1μl each of 10 pmol/μl primers (11f and 1392r), 0.5μl of 5U/μl Taq PCR polymerase (Takara co. Japan) and 2μl of diluted DNA template were added to a 200μl tube. The total volume was adjusted to 100μl with sterilized ddH_2O. The PCR was performed with the GeneAmp PCR System 9600 (Perkin Elmer Co.) with the following thermal program: initial denaturation (95°C for 3 min), 30 cycles of amplification

(denaturation at 95°C for 1.5 min, annealing at 52°C for 45 s, and extension at 72°C for 2 min), and a final extension (72°C, 5 min). The PCR product was checked by electrophoresis in 1 % (wt/vol) agarose gel and purified with the StrataPrep PCR purification kit.

For cloning library construction, the amplified PCR product was ligated into the pPCR-Script Amp SK(+) cloning vector (Stratagene Co.) according to the manufacturer's instruction. Clones containing correctly rDNA inserts were checked by PCR amplification M13f/M13r PCR products. The primer sequences used in this study were showed in Table 1. Clones with the correctly insertion of 16S rDNA gene were screened by denaturing gradient gel electrophoresis (DGGE) as previously described [24]. The DGGE was performed with a Biorad Dcode system at 200V for 4hr. The DGGE patterns were examined after SYBR Green I staining. Based on the electrophoresis on the DGGE gel, clones with unique DGGE electrophoresis position were obtained and sequenced with primer 11f and 1392r by using a DNA auto-sequencer (ABI model 377A).

Table 1. The primer sequence of PCR

Primer	Sequence	Reference
11f	5'-GTTTGATCCTGGCTCAG-3'	26
1392r	5'-ACGGGCGGTGTGTAC-3'	24
M13 (-20)	5'-GTAAAACGACGGCCAG-3'	27
M13r	5'-CAGGAAACAGCTATGAC-3'	27
968fgc	5'-CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGG GGAACGCGAAGAACCTTAC-3'	24

Table 2. Oligonucleotide probes used in this study

Probe	Specificity	Sequence (5'-3') of probe	F.A. (%)	Target site	Reference
EUB338	Bacteria	GCTGCCTCCCGTAGGAGT	25	16S (338-355)	28
ALF1b-a	α-subdivision of *Proteobacteria*, several members of delta subdivision of *Proteobacteria*, most spirochetes	CGTTCGCTCTGAGCCAG	25	16S (19-35)	29
BET42a	β-subdivision of *Proteobacteria*	GCCTTCCCACTTCGTTT	35	23S (1027-1043)	29
GAM42a	γ -subdivision of *Proteobacteria*	GCCTTCCCACATCGTTT	35	23S (1027-1043)	29
HGC69a	Gram positive high G+C phylum	TATAGTTACCACCGCCCT	25	23S (1901-1918)	30
CF319a	*Cytophagales*	TGGTCCGTGTCTCAGTAC	35	16S	29
BTWO23a	Competitor of BONE23a	GAATTCCACCCCCCTCT	35	16S (663-679)	31
BONE23a	β-1 group of *Proteobacteria*	GAATTCCATCCCCCTCT	35	16S (663-679)	31
LDI23a	*Leptothrix discophora* and other members of β-1 group of *Proteobacteria*	CTCTGCCGCACTCCAGCT	35	16S (649-666)	32

2.3. Phylogenetic analysis

The phylogenetic affiliation of the clones from DGGE screened was analyzed. Sequences of the most 10 abundance 16S rDNA clones were compared to the available 16S rRNA sequences in GenBank. The most closely related 16S rRNA sequences from the search in the GenBank were aligned with a sequence alignment editor, ARB program [25].

2.4. *In-situ* hybridization

The sample were fixed for 3 hr with 4% paraformaldehyde as described by Amann *et al.* [26] and stored in a 1:1 mixture of phosphate-buffered saline (PBS, pH 7.4) and 96% ethanol at –20°C. Cells were immobilized onto poly-L-lysine coated slides and dehydrated by sequential washes in 50, 80, and 100% ethanol for 3 min, respectively. Hybridization and washing procedures were carried out according to the protocol previous described [24]. Fluorescent hybridized cells were analyzed with a Zeiss Axioscop2 epifluorescence microscope equipped with a cooled CCD camera (Quntax; Photometrics, USA). The oligonucleotide probes and stringency used in this study were listed in Table 2.

3. RESULTS AND DISCUSSION

3.1. Microbial community structure based on 16S rDNA clone library

161 clones containing correct inserts of 16S rDNA were obtained from the clone library construction and were further amplified with DGGE-PCR primer set. After screened based on the electrophoresis position on a DGGE gel, these clones were subsequently classified into 34 different operational taxonomic units (OTUs) (i.e., different electrophoresis position). The 10 most abundance OTUs, which represented about 82.6% of total clones, were further sequenced and compared with the available 16S rRNA sequences in GenBank. Table 3 indicates that these OTUs were separately affiliated with the α- (1 OTU, 7.5% of total clones) and β- (5 OTUs, 49.7%) subdivisions of the division *Proteobacteria*, and *Cytophaga* (4 OTUs, 25.5%). The closest related sequence and the similarity of individual OTUs were also shown in Table 3.

Those five OTUs (Tu7, Tu8, Tu1, Tu37, and Tu81) obtained within the beta-*Proteobacteria* were all affiliated within the β-1 subdivision [33]. The exact phylogenetic placement of these five OTUs was examined by constructing a phylogenetic tree as shown in Figure 1. This phylogeny tree indicates that at least five known clusters as described by Snaidr *et al.* [34] are affiliated within in the β-1 *Proteobacteria*. OTUs tu1 and tu8 could be successfully assigned to cluster II (i.e., the genus *Acidovorax*) and the genus *Hydrogenophaga*, respectively. But, the other three OTUs (34.1% of total clones) were found to form a new cluster with no known bacterial isolates, and were relatively related to cluster I.

Recent studies supported delta-*Proteobacteria* as a major population in activated sludge processes [12, 34-36]. The study of Snaidr and co-worker [34] further revealed that a large fraction of clones found in a full-scale wastewater treatment plant was from the β-I *Proteobacteria*, but were associated within clusters I to V. However, little operational information provided for the full-scale wastewater treatment plant has increased the difficulty in the comparison of microbial community structure [34]. Furthermore, members of the β-I *Proteobacteria* that were found in this study were different from those previously obtained from deteriorated EBPR processes. Bond *et al.* [36] recently reported that members within the

β-*Proteobacteria* but other than β-I and β-II subdivisions were predominant in a deteriorated EBPR process fed with acetate. Furthermore, Nielsen *et al.* [24] reported that a novel group in the γ-*Proteobacteria* was dominant in a deteriorated EBPR process fed with acetate. The difference in the predominant population among these studies was very encouraging. It is clear that members of GAOs were composed by at least three populations from different phylogenetic groups. The predominance of different GAO populations was possibly attributed to the difference in operational conditions (e.g., continuous and sequential) and substrate composition (e.g., acetate and fermented products) in different activated sludge processes. Since the cluster represented by OTU tu7 was likely a novel cluster within the β-1 subgroup, its distribution and metabolic function in various EBPR processes warrants further study.

In addition, a significant fraction of clones was affiliated with *Cytophaga*. Table 3 indicates that most of the OTUs were not closely related (<98% in sequence similarity) to any known bacteria. Three OTUs (Tu72, Tu70 and Tu74) were affiliated with the genus *Flavobacterium*. OTUs tu72 was affiliated with a filamentous bacterium, *Haliscomenobacter hydrossis*. Even though, clones related to *Cytophaga* have been constantly found in activated sludge processes [32, 35], the metabolic role of this microbial group in activated sludge was hardly understood.

3.2. *In-situ* hybridization of predominant populations

Cloning analysis revealed that microbial populations in the β-subdivision of the *Proteobacteria, Cytophagales* division, and *alpha* subdivision of the *proteobacteria* were the predominant ones. Thus, the predominance of these major phylogenetic groups in the activated sludge was further verified using *in-situ* hybridization with 16S rRNA-targeted oligonucleotide probes. These probes included ALF1b-a, BET42a, GAM42a, CF319a, and

Table 3. The phylogenic affiliation of the most abundant clones obtained in activated sludge.

Clone no.	Length of Sequence (bases)	Phylogenetic relationship: Species (source in genebank)	Phylogenetic ffiliation*	% of Similarity	Abundance of clones (%)
Tu7	1399	Activated sludge clone T70	β	95	26.6
Tu72	1377	*Haliscomenobacter hydrossis*	*Cytophagales*	95	11.8
Tu8	1390	*Hydrogenophaga taeniospiralis*	β	98	8.7
Tu23	1006	Activated sludge clone MK09	*Cytophagales*	88	8.1
Tu27	1337	*Acidosphaera rubifaciens*	α	93	7.5
Tu1	1383	Activated sludge isolate rJ12	β	99	6.9
Tu37	1379	Activated sludge clone T41	β	98	5.6
Tu70	1387	Activated sludge clone 49511	*Cytophagales*	97	3.7
Tu74	1378	*Flavobacterium aquatile*	*Cytophagales*	94	1.9
Tu81	1379	Activated sludge clone T41	β	98	1.9
	Other 20 species which the clones number were less than 2 (total 28clones)				16.1
				Total	100

*β, beta-subdivision of *Proteobacteria*; α, alpha-subdivision of *Proteobacteria*.

HGC69a that were specific for α-, β-, and γ-subdivisions of the division *Proteobacteria*, *Cytophaga*, and the gram-positive with high G+C DNA content bacterial group, respectively. As Figure 2 shown, the β-subdivision of the division *Proteobacteria* dominated at least 80% of the bacterial population, followed by α-*Proteobacteria*. Figure 3 further indicates that probe BET42a strongly hybridized with coccoid or tetrad cells (panels A and B) that usually formed dense flocs in the sludge sample (panel C). Especially, the tetrad cells were often observed to be the predominant populations in EBPR processes during deterioration [8]. In this study, PHB staining [37] performed on the sludge sample further showed positive response with the clustered cells including the tetrad cells (data not shown).

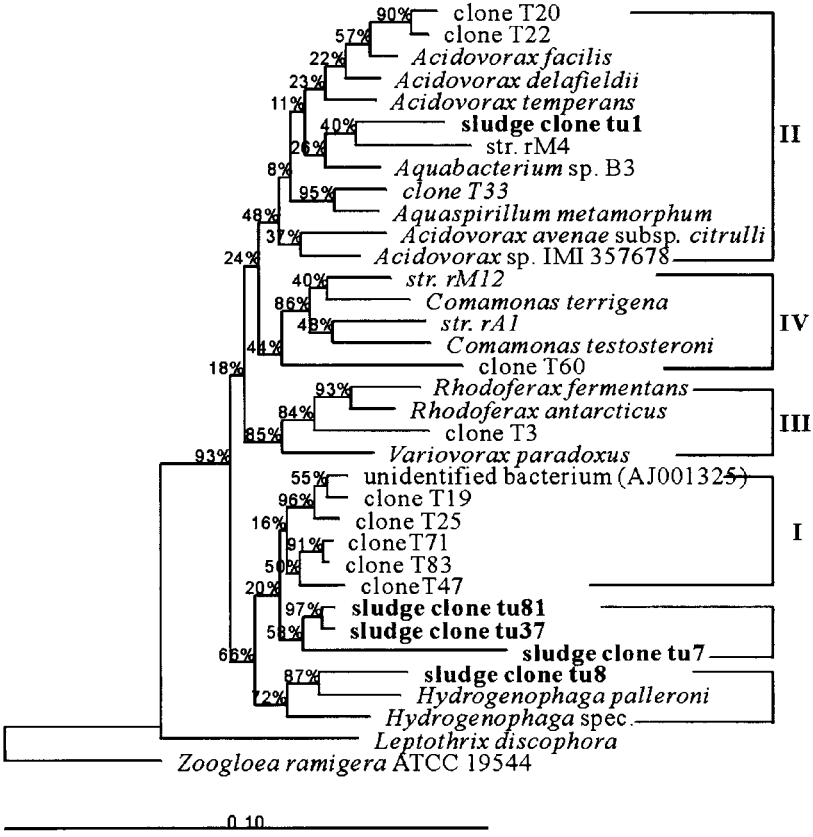

Figure 1. Phylogenetic tree showing the relationship of the obtained sludge 16S rDNA clones and their closest relatives among the β-I group members of *Proteobacteria*. The distant matrix tree was constructed using the neighbor-joining algorithm with bootstrapping (100 times). Those clusters numbered were the same as described by Snaidr *et al.* [34]. The bar scale indicates 10 nucleotide substitutions per 100 nucleotides.

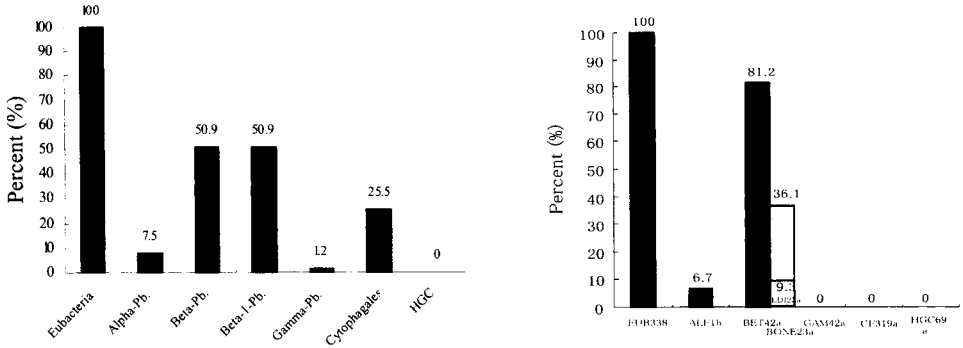

Figure 2. The community abundance as determined by cloning analysis (a) and in situ hybridization (b). Alpha-*Pb.*, Beta-*Pb.*, and Gamma-*Pb.*, the α, β, and γ-subclass of *Preteobacteria*; Beta-*1-Pb.*, the β-I subdivision of *Preteobacteria*; *Cytophagales*, *Cytophagales* class.

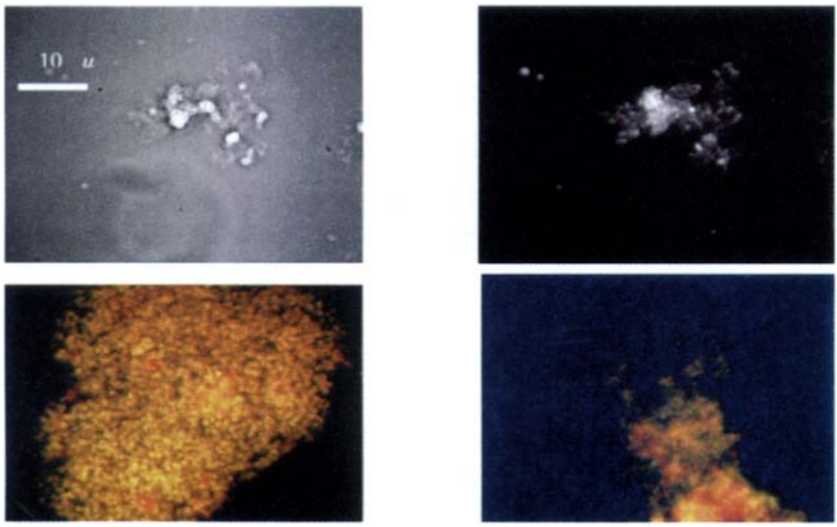

Figure 3. The microbial community of the deteriorated EBPR process as revealed by *in-situ* hybridization with different oligonucleotide probes. Panels (a) and (b) show the predominance of clustered tetrad cells under phase-contrast observation and in-situ hybridized with BET42a, respectively. Panel (c) shows the combined *in-situ* hybridization of activated sludge with cy5-labled BET42a (green) and cy3-labled EUB338 (red) probes. Cells simultaneously hybridized with two probes were shown in yellow. Panel (d) shows the combined in-situ hybridization with cy5-labeled BONE23a (red) and cy3-labled EUB338 (green) probes.

Probes BONE23a and LDI23a were further used to determine the distribution of microbial populations within the β-I subdivision of *Proteobacteria*. Results (Figure 2) indicate that a major fraction of cells within the β-subdivision was hybridized by these two probes. One of the cells hybridized by probe BONE23a was a coccoid cell, which was usually present inside microbial clusters. Probe LDI23a that mainly targeted the genus *Leptothrix* only hybridized with a relative small fraction of cells.Cells in β-*Proteobacteria* but not detected by BONE23a suggested the presence of microbial populations other than β-I subdivision in the activated sludge. This result supported the finding of the cloning analysis that microbial populations associated within β-I subdivision were the predominant population.

In-situ hybridization of activated sludge with the α-*Proteobacteria* probe (ALF1b-a) only detected a small fraction of cells (data not shown). This result was allied with the cloning analysis. No hybridization signals were observed with probes HGC69a and CF319a in the *in-situ* hybridization. Especially, clones related to the *Cytophaga* accounted for at least 25.5% of total clones, and their sequences were complementary to probe CF319a. One possible reason led to the overestimation of the *Cytophaga* population in the cloning analysis was associated with biases occurring in the steps of DNA extraction, PCR amplification and cloning [34].

In summary, the microbial community of a non-phosphate removing anaerobic-aerobic bioreactor fed with fermented products was predominant by microbial populations associated with the β-I subgroup of the *Proteobacteria* (57.2%) and *Cytophagales* (25.5%). In particular, a novel bacterial cluster that was closely related to the genus *Hydrogenophaga* was the predominant population. It was known that the phylogenetic information does not reflect the physiological status of a microbial population in microbial ecosystems. Hence, the methods used in this study was insufficient to verify that the microbial population, especially the novel cluster group in the β-I subgroup, was responsible for the metabolism observed in the process. In our further experiment, a simple approach that combine the PHB stain with in-situ hybridization will be used to link functional traits to a phylogenetic population in the deteriorated EBPR sludge. Another promising approach will be the combination of microautoradiography and in-situ hybridization [38].

REFERENCE

1. Y. Comeau, K. J. Hall, R. E. W. Hancock, and W. K. Oldham, Wat. Res., 27 (1993) 1219.
2. T. Mino, M. C. M. van Loosdrecht, and J. J. Heijnen, Water. Res., 32 (1998) 3193.
3. T. Fukase, M. Shibata and Y. Miyaji, Water Sci. Tech., 17 (1985) 68.
4. D. Brdjanovic, C. M. Hooijmans, M. C. M. van Loosdrecht, G. J. Alaerts and J. J. Heijnen, Water Res., 30 (1996) 2323.
5. D. Brdjanovic, A. Slamet, M. C. M. van Loosdrecht, C. M. Hooijmans, G. J. Alaerts and J. J. Heijnen, Water Res., 32 (1998) 200.
6. T. Kuba, A. Wachtmeister, M. C. M. Loosdrecht and J. J. Heijnen, Water Sci. Tech., 30 (1994) 263.
7. J. B. Cech and P. Hartman, Water Res., 27 (1993) 1219.
8. W. T. Liu, T. Mino, T. Matsuo and K. Nakamura, Wat. Res., 30 (1996) 75.
9. J. B. Cech and P. Hartman, Environ.Technol., 11 (1990) 651.
10. W. T. Liu, K. Nakamura, T. Mino, and T. Matsuo, Wat. Res., 31 (1997) 1430.
11. Y. Matsuo, Water Sci. Tech., 28 (1994) 127.

12. P. L. Bond, P. Hugenholtz, J. Keller, and L. L. Blackall, Appl. Environ. Microbiol., 61 (1995) 1910.
13. L.L. Blackall, P. C.Burrell, H. Gwilliam, D. Bradford, P. L. Bond and P.Hugenholtz, Water. Sci. Tech., 37 (1998) 451.
14. A. Teske, C. Wawer, G. Muyzer, and N. B. Ramsing, Appl. Environ. Microbiol.,62 (1996) 1405.
15. A. Teske, P. Sigalevich, Y. Cohen, and G. Muyzer, Appl. Environ. Microbiol.,62 (1996) 4210.
16. C. M. Santegoeds, S. C. Nold, and D. de Beer, Appl. Environ. Microbiol.,62 (1996) 3922.
17. M. J. Ferris and D. M. Ward, Appl. Environ. Microbiol., 63 (1997) 1375.
18. G. A. Kowalchuk, J. R. Stephen, W. de Boer, J. I. Prosser, T. M. Embley, and J. W. Woldendrop, Appl. Environ. Microbiol., 63 (1997) 1489.
19. M. Wagner, R. Erhart, W. Manz, R. Amann, H. Lemmer, D. Wedi and K. H. Schleifer, Appl. Environ. Microbiol, 60 (1994) 792.
20. M. Wagner, R. Amann, H. Lemmer, W. Manz, and K. H. Schleifer, Wat. Sci. Tech., 29 (1994) 15.
21 W.-T Liu, K. Linning, K. Nakamura, T. Mino, T. Matsuo, L.J. Forney, Microbiology (2000) in press.
22. T. Nomura, T. Mino and T. Matsuo, unpublished data (1999).
23. H. Zhu, F. Qu, and L. H. Zhu, Nucleic Acids Res., 21 (1993) 5279.
24. A. T. Nilsen, W. T. Liu, C. Filipe, L. Grady, J. S. Molin and D. A. Stahl, Appl. Environ. Microbiol, 65 (1999) 1251.
25. O. Strunk, O. Gross, B. Reichel, M. May, S. Hermann, N. Stuckman, B. Nonhoff, M. Lenke, A. Ginhart, A, Vilbig, T. Ludwig, A. Bode, K. H. Schleifer and W. Ludwig, in submission, 1997.
26. R. I. Amann, W. Ludwig, and K. H. Schleifer, Microbiol. Rev., 59 (1995) 143.
27. C. Yanish-Person, J. Viera, and J. Messing, Gene, 33 (1985) 103-199.
28. R. I. Amann, L. Krumholz, D. A. Stahl, J. Bact., 172 (1990) 762.
29. W. Manz, R. I. Amann, W. Ludwig, M. Wagner, and K. H. Schleifer, Syst. Appl. Microbiol., 15 (1992) 593.
30. C. M. Roller, M. Wagner, R. Amann, W. Ludwig, and K. H. Schleifer, Microbiology, 140 (1994) 2849.
31. R. I. Amann, J. Snaidr, M. Wagner, W. Ludwig, and K. H. Schleifer, J. Bact., 178 (1996) 3496.
32. M. Wagner, R. Amann, P. Kampfer, B. Assmus, A. Hartmann, P. Hutzler, N. Springer, and K. H. Schleifer, Syst. Appl. Microbiol., 17 (1994) 405.
33. C. R. Woese, Microbiol Rev., 51 (1987) 221.
34. J. Snaidr, R. Amann, I. Huber, W. Ludwig and K. H. Schleifer, Appl. Environ. Microbiol, 63 (1997) 2884.
35. W. Manz, M. Wagner, K. Amann, K. H. Schleifer, Wat. Res., 28 (1994) 1715
36. P. L. Bond, R. Erhart, M. Wagner, J. Keller. L. L. Blackall, Appl. Environ. Microbiol., 65 (1999) 4077.
37. D. Jenkins, M. G. Richard, and G. T. Daigger, Manual on the causes and control of activated sludge bulking and forming, 2nd edn, Lewis Publishers, Michigan (1993).
38. N. Lee, P.H. Nielsen, K.H. Andreasen, S. Juretschko, J.L. Nielsen, K.-H. Schleifer, and M. Wagner, Appl. Environ. Microbiol. 65 (1999) 1289.

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Microbial aspects of autotrophic denitrification of wastewaters*

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For wastewaters with a low BOD_5/N ratio, autotrophic denitrification using *Thiobacillus denitrificans* is an interesting alternative to heterotrophic denitrification. In this paper, investigations on the application of autotrophic denitrification in elemental sulphur packed bed reactors to nitrified leachate, secondary effluent, and flue gas desulphurisation wastewater are summarised. Special emphasis is given to the effect of environmental and operational variables on microbial activity.

1. INTRODUCTION

Biological nutrient removal has become an integrated part of modern wastewater treatment. For wastewaters with a low BOD_5/N ratio, autotrophic denitrification using *Thiobacillus denitrificans* is an interesting alternative to heterotrophic denitrification. This autotrophic bacteria oxidises reduced sulphur compounds such as elemental sulphur or thiosulphate to sulphate while reducing nitrate to elemental nitrogen gas, thereby eliminating the need for addition of organic carbon sources. The stoichiometric reaction of autotrophic denitrification using elemental sulphur can be represented by the following equation [1]:

$$NO_3^- + 1.10\ S + 0.40\ CO_2 + 0.76\ H_2O + 0.08\ NH_4^+ \xrightarrow{\textit{Thiobacillus denitrificans}} 0.5\ N_2 + 1.10\ SO_4^{2-} + 1.28\ H^+ + 0.08\ C_5H_7O_2N \quad (1)$$

Contrary to heterotrophic denitrification, autotrophic denitrification consumes alkalinity and, in addition, generates high concentrations of sulphate. Fortunately, this does not pose an undue problem in coastal areas such as Hong Kong where wastewater can be discharged directly to the sea, which has a natural sulphate concentration of 2,700 mg/l.

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Autotrophic denitrification has been extensively investigated to remove nitrate from polluted groundwater (e.g. Kruithof *et al.*, 1988[2]; Gayle *et al.*, 1989[3]; Hiscock *et al.*, 1991[4]; Kapoor and Viraraghavan, 1997[5]). However, little research has been conducted on autotrophic denitrification of raw or pre-treated wastewater with low BOD_5/N ratios, nor on the effect of major operational and environmental variables on it. Septic effluent, nitrified leachate, ion exchange resin eluates, and a few synthetic wastewaters have been studied, but in most cases thiosulphate was used as the reduced sulphur source [6-9].

In this paper, some applications of autotrophic fixed-film denitrification using elemental sulphur are summarised, with special emphasis given to the effect of environmental and operational variables on microbial activity. Wastewaters investigated included nitrified leachate, secondary effluent, and flue gas desulphurisation (FGD) wastewater.

2. MATERIALS AND METHODS

2.1. Experimental apparatus

Three identical packed bed reactors were used to carry out fixed-film denitrification of nitrified landfill leachate. They had an inside diameter of 84 mm and were packed with small elemental sulphur particles to a height of 1650 mm. Three sulphur particle size ranges were employed, namely 2.8 to 5.6 mm, 5.6 to 11.2 mm, and 11.2 to 16 mm, respectively. The reactors were fed continuously in the upflow mode by means of adjustable peristaltic pumps. All experiments were conducted at ambient temperature of approximately 25 ^{0}C. In the case of secondary effluent, the sulphur packing height was reduced to 390 mm because of the relatively low nitrate concentration, and only the small sulphur particle size of 2.8 to 5.6 mm was used. A schematic diagram of the continuous flow packed bed reactor system is shown on Figure 1.

For the determination of the effect of environmental variables or potentially inhibiting substances, especially in connection with the feasibility study on autotrophic denitrification of FGD wastewater, standardised batch tests were employed whereby 250 mL flasks containing given quantities of sulphur particles and microbial population were incubated under controlled conditions.

2.2. Experimental program

The preparation and enrichment of *Thiobacillus denitrificans* cultures has been described

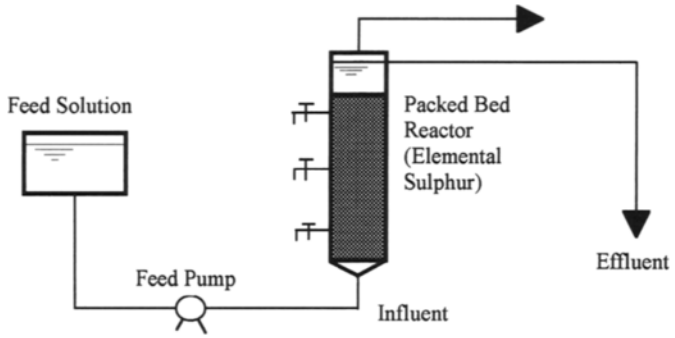

Figure 1. Schematic diagram of continuous flow packed bed reactor system

previously [10]. After sufficient development, the enriched cultures were introduced into the packed bed reactors for attachment to the sulphur particles and formation of a biofilm. A series of tests were carried out whereby the hydraulic retention time was gradually decreased until the reactors became overloaded, resulting in a drastic decrease of nitrate removal efficiency. Each hydraulic retention time was maintained as long as was necessary to achieve steady-state conditions. All reactors were sampled daily. Analyses were carried out for nitrate, nitrite, pH, alkalinity and sulphate according to *Standard Methods* (1989)[11]. Additional parameters such as more detailed wastewater constituents or gas production/composition were determined as needed. In the standardised batch tests, the relevant parameters indicating microbial activity were determined in thirty-minute intervals.

2.3. Source and characteristics of wastewater

Nitrified leachate was obtained from the leachate treatment works at the Hong Kong NENT Strategic Landfill where leachate is biologically treated and fully nitrified in aerated lagoons. Secondary effluent was obtained from the two government operated Sha Tin and Tai Po BNR Sewage Treatment Works. The FGD wastewater originated from the FGD wastewater treatment plant of a local electric power station. When required, the wastewaters were diluted with tapwater to the desired NO_3-N concentration and an appropriate amount of $NaHCO_3$ was added for provision of sufficient buffer capacity. For comparative studies, synthetic wastewater was also prepared. It consisted of a solution of tap water containing approximately 30 mg/l KNO_3 (as N), 500 mg/l $NaHCO_3$, 1 mg/l K_2HPO_4 (as P), 0.5 mg/l NH_4Cl (as N), 0.5 mg/l $MgCl_2.6H_2O$ and 0.5 mg/l $FeSO_4.7H_2O$. For higher concentrations of NO_3-N, the concentrations of KNO_3 and $NaHCO_3$ were correspondingly increased. For tests on the effects of salinity, appropriate amounts of NaCl or Na_2SO_4 were added. The typical composition of the actual wastewaters used for this research is shown in Table 1. Moderate to high salinity in all wastewaters is noticeable.

3. RESULTS AND DISCUSSION

3.1. Removal efficiencies and operational factors

Results on the feasibility of autotrophic denitrification for nitrified leachate and secondary effluent have been published previously [10,12-14]. It could be demonstrated that *Thiobacillus*

Table 1
Typical composition of wastewaters used

		Secondary effluent		Nitrified	FGD
		Tai Po STW	Sha Tin STW	leachate	wastewater
COD	mg/L	50	46	1153	35
BOD_5	mg/L	3.3	3.3	12	<5
NH_4^+-N	mg/L	0.4	3.2	5.1	n.d.
NO_3^--N	mg/L	5.6	3.1	1665	330
NO_2^--N	mg/L	0.06	1.2	1.1	n.d.
PO_4-P	mg/L	2.2	1.6	1.9	0.003
SO_4^{2-}	mg/L	690	765	99	5500
Cl^-	mg/L	4680	4970	5300	13300
Alkalinity	mg/L $CaCO_3$	90	117	1058	1091
pH	-	7.5	7.7	8.1	8.5
BOD_5/NO_x-N ratio		0.58	0.77	0.007	0

denitrificans can effectively remove nitrate in sulphur packed beds from concentrations of up to 800 mg/L NO_3-N to a concentration of less than 1 mg/L. The minimum retention time required for complete denitrification depends on sulphur particle size and influent nitrate concentration. For example, using a sulphur particle size of 2.8 to 5.6 mm, nitrified leachate with 800 mg/L NO_3-N required a minimum hydraulic retention time of 11.4 hours, while 5.7 hours were sufficient to completely denitrify 400 mg/L NO_3-N. Similarly, it could be shown that for a given influent concentration the minimum retention time was inversely proportional to the mean sulphur particle size. The shallow packed bed reactors for secondary effluents did not perform as well as the deep packed bed reactors, possibly due to less than ideal flow conditions in shallow beds. For example, using a sulphur particle size of 2.8 to 5.6 mm, synthetic secondary effluent with 32 mg/L NO_3-N required a minimum hydraulic retention time of 4.0 hours. It should be noted, that in the above the hydraulic retention times are expressed as bed pore volume divided by influent feed flow rate.

3.2. Effect of environmental and operational variables: temperature, pH, salinity, nutrients, inhibiting substances, alkalinity

Figures 2 to 4 show the results of batch test studies on the effects of temperature, pH, and salinity (expressed as osmotic pressure) on the autotrophic denitrification rate. It appears that *Thiobacillus denitrificans* is quite sensitive to temperature, with 30 ^{0}C being the optimum. The denitrification rate follows the well-known Arrhenius relationship up to a temperature of 30 ^{0}C. The activation energy E_a was determined as 53.2 kJ/mole, which results in a Q_{10} value of 2.05 indicating a nearly perfect doubling of the reaction rate for a 10 ^{0}C temperature increase. Batchelor and Lawrence (1978)[1] reported an almost identical value of 54.4 kJ/mole for the activation energy, but did not determine the optimum temperature. The highest rates of autotrophic denitrification were observed within the range of pH 7 to 8. For growth of pure *Thiobacillus denitrificans* cultures using thiosulphate, comparable results with an optimum temperature of 25 to 30 ^{0}C and an optimum pH of 6 to 8 were reported [15-17]. It may be of interest to note that the effects of temperature and pH on heterotrophic denitrification rates, which were established much earlier, exhibit similar patterns [18].

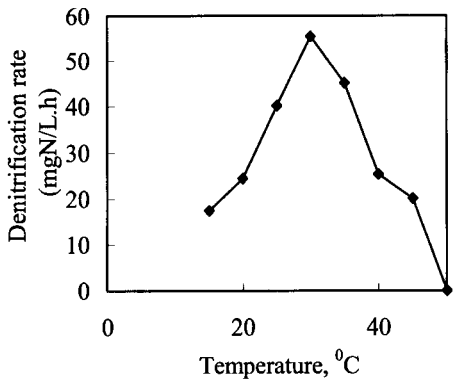

Figure 2. Effect of temperature on denitrification rate

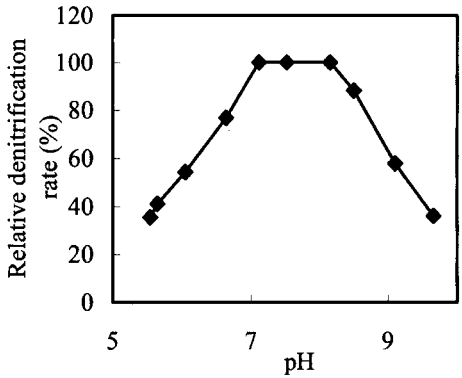

Figure 3. Effect of pH on denitrification rate

Due to the high salinity of the wastewaters used in this research, the effect of salinity was

carefully investigated. It is known that the osmotic pressure of dissolved salts or particles in a solution affects the movement of substances across the cell membrane of bacteria and hence their activity. Osmotic pressure is directly related to the molar concentration of dissolved salts or particles and can be approximately calculated according to the van't Hoff equation. From Figure 4 it is apparent that denitrification rates start to decrease above an osmotic pressure of about 19 atm, independent of the type of salt used. At the osmotic pressure of seawater, equal to 26.9 atm for the 1.1 molar solution of anions and cations, the denitrification rate is reduced to about 70% of the maximum rate. Glass and Silverstein (1999)[19] surveyed the literature on heterotrophic denitrification under highly saline conditions and found no consensus, but several studies indicated successful denitrification at 30 g/L NaCl.

Nutrient absence or deficiency may depress the denitrification rate. Figure 5 demonstrates the effect of phosphate addition on the autotrophic denitrification of FGD wastewater. Addition of phosphate (as K_2HPO_4) in this case is quite difficult, since the high calcium content of 2600 mg/L in FGD wastewater leads almost immediately to a precipitation of calcium phosphate. The calcium had therefore to be removed by use of sodium carbonate; alternatively, addition of polyphosphate $(NaPO_3)_n$ was able to prevent precipitation, but its metabolic availability to the bacteria was not known. Fortunately, both methods produced satisfactory results.

No information on the effect of inhibitory substances on autotrophic denitrification could be found in the literature. The unusually high concentration of boron in FGD wastewater caused concern about a possible inhibitory effect. However, such effect was manifested only above concentrations of 500 mg/L, which are rarely found in wastewaters.

Equation (1) predicts that for each mg NO_3-N removed about 4.5 mg alkalinity (as $CaCO_3$) is consumed. For high nitrate concentrations, the associated high consumption of alkalinity in the wastewater could result in a dramatic pH drop when buffer capacity is insufficient. The

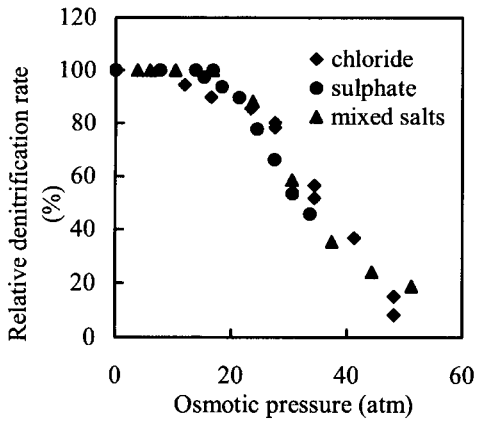

Figure 4. Effect of osmotic pressure on denitrification rate

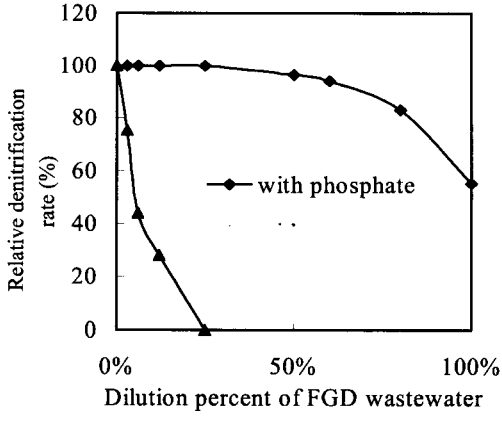

Figure 5. Effect of nutrient addition on denitrification rate

secondary effluents used in this study provided more alkalinity than required. For nitrified leachate, some alkalinity in the form of $NaHCO_3$ had to be supplemented; alternatively, addition of limestone granules to the packed sulphur bed prevented a pH drop below 6.5. However, in the batch tests with FGD wastewater, serious problems were encountered to raise the alkalinity since addition of $NaHCO_3$ caused precipitation of $CaCO_3$ because of the high calcium content in the wastewater. Prior to $NaHCO_3$ addition, the calcium had therefore to be removed by precipitation with sodium carbonate. It remains to be seen whether limestone addition in the packed bed reactors will eliminate the alkalinity problem and avoid the need for physical-chemical pretreatment of FGD wastewater to remove calcium. Since heterotrophic denitrification is known to generate alkalinity, simultaneous heterotrophic and autotrophic denitrification has been proposed to maintain alkalinity [7,8]. However, more than 56% of NO_3^--N should be denitrified by heterotrophic denitrification using methanol addition to eliminate the need for alkalinity addition.

Buildup and fouling of biomass may be encountered in packed bed reactors. No such problems were observed when the influent was nitrified leachate or synthetic wastewater. However, the outflow of the secondary effluent emitted a noticeable H_2S odour after several weeks of operation. Sulphide concentrations between 0.3 and 2.2 mg/L (as S^{-2}) confirmed the existence of less than ideal anoxic conditions in the reactor. Possible causes for biofouling were the presence of SS and COD in the secondary effluent, which could have led to additional microbial growth and partly anaerobic conditions due to degradation. More frequent backwashing and application of ORP control may be required to avoid biofouling, since ORP values below –200 mv have been associated with the completion of denitrification and onset of anaerobic conditions as indicated by sulphide formation (Charpentier *et al.*, 1998)[20]. Similar differences in biofouling were reported for autotrophic denitrification of real and synthetic groundwater (Flere and Zhang, 1999)[21].

3.3. Microbial kinetics

It could be demonstrated [22] that autotrophic denitrification in sulphur packed bed reactors follows a half order reaction according to biofilm theory, first proposed by Harremoes (1976)[23] for heterotrophic packed bed denitrification:

$$C_1^{1/2} = C_0^{1/2} - \tfrac{1}{2} k_{1/2v} t \qquad (2)$$

where C_0 = influent concentration, in mg/L NO_3-N; C_1 = effluent concentration, in mg/L NO_3-N; t = empty bed residence time, in hours; and, $k_{1/2v}$ = half-order reaction rate constant per unit reactor volume, in $mg^{1/2}/L^{1/2}.h$. This equation can also be used to estimate the nitrate profiles in the packed bed reactor, when t is taken as the empty bed residence time in the reactor up to a given height. It could be further shown that the half-order reaction rate constant $k_{1/2v}$ depends on the specific surface area of the reactor media and hence the sulphur particle size according to the following relationship:

$$k_{1/2v} = 0.0419\omega \qquad (3)$$

where ω = specific surface area of the reactor media, i.e. of the sulphur particles, per unit reactor volume, in dm^2/dm^3. The constant 0.0419, in $mg^{1/2}/dm^{1/2}.h$, corresponds to $k_{1/2a}$, which is the half-order reaction rate constant per unit biofilm area. Compared to heterotrophic

denitrification, the half-order reaction rate constant $k_{1/2a}$ for autotrophic denitrification was found to be lower by approximately one order of magnitude.

The maximum specific denitrification rate was found to be about 0.15 g NO_3-N/g VSS.d at 25 ^{0}C, comparable to values of 0.1 and 0.2 g NO_3-N/g VSS.d estimated from the data of Batchelor and Lawrence (1978)[1] in a suspended growth system. These values are again approximately one order of magnitude lower than those reported for heterotrophic denitrification of wastewater, which amount to approximately 2.65 g NO_3-N/g VSS.d based on kinetic coefficients reported by Henze *et al.* (1997)[24]. However, when using thiosulphate instead of elemental sulphur as electron donor, the maximum specific denitrification rates of pure *Thiobacillus denitrificans* cultures in chemostats were estimated as 4.23 and 8.40 g NO_3-N/g VSS.d, respectively [15,17], comparable to 4.41 g NO_3-N/g VSS.d for heterotrophic denitrification using methanol as electron donor [24]. The exact reason for the low denitrification rate when using elemental sulphur was not determined, but it is known that the energy yield in autotrophic denitrification when using elemental sulphur or thiosulphate is almost the same at 91.15 and 92.97 kJ/electron equivalent, respectively [25]. Besides, for heterotrophic denitrification using methanol, the energy yield is only slightly higher at 109.18 kJ/electron equivalent. It appears therefore likely, that the limiting factor for the denitrification rate when using elemental sulphur is the slow dissolution and transport of elemental sulphur in the biofilm, and not any intrinsically lower energy yield or reaction rate for the autotrophic denitrification reaction.

An interesting phenomenon observed was the formation of deep holes on the surface of the sulphur granules after a long period of operation (Figure 6). Since the sulphur particles serve not only as media for attached growth, but also as the source of energy, this growth pattern of *Thiobacillus denitrificans* seems to increase the specific surface area for maximisation of sulphur dissolution and hence the denitrification rate of the bacteria. The mechanism for the hole formation is not clear. Conventional biofilm theory does not consider this phenomenon and assumes uniform biofilm thickness as well as biologically inert media.

3.4. Metabolic pathway

No information on the specific metabolic pathway of autotrophic denitrification by *Thiobacillus denitrificans* could be found in the literature. It was observed that for complete denitrification practically all the NO_3^--N was converted to elemental nitrogen gas N_2, with no intermediate products detected. However, when the reactors became overloaded, NO_2^--N rapidly built up in the effluent, while nitrous oxide gas N_2O was found in the waste gas. However, N_2O-N in the waste gas never exceeded 0.8% of the influent NO_3^--N, which is quite low in comparison to the other forms of nitrogenous products, namely N_2 and NO_2^-. Autotrophic denitrification by *Thiobacillus denitrificans* seems therefore to proceed in a similar manner as heterotrophic denitrification according to the following enzymatically mediated steps: $NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$. The rate limiting steps are most likely $NO_2^- \rightarrow N_2O$ and $N_2O \rightarrow N_2$ which would explain the accumulation of NO_2^- and N_2O.

Besides N_2, the waste gas sometimes contained unexpectedly high concentrations of CO_2. The reason for this was purely physico-chemical, as it could be shown that the CO_2 concentration in the waste gas is in equilibrium with the pH and alkalinity of the denitrified effluent according to Henry's Law. This is analogous to the CO_2 concentration in the digester gas being in equilibrium with the pH and alkalinity of the supernatant.

3.5. Stoichiometric relationships

The following equation corresponds most closely to the experimentally found stoichiometric relationships between NO_3-N removed, alkalinity consumed, and nitrogen gas and sulphate formed:

$$1.06\ NO_3^- + 1.11\ S + 0.30\ CO_2 + 0.785\ H_2O \xrightarrow{\textit{Thiobacillus denitrificans}} 0.5\ N_2 + 1.11\ SO_4^{2-} + 1.16\ H^+ + 0.06\ C_5H_7O_2N \quad (4)$$

The results obtained compare well with the findings of Batchelor and Lawrence (1978) although they used a suspended growth system instead of packed bed reactors. According to Equation (1), nitrogen in the form of ammonia is required for microbial synthesis, amounting to 8% by mass of the nitrate nitrogen to be denitrified. However, after a suitable period of adaptation, the packed bed reactors functioned quite well without supplementary addition of ammonia nitrogen. This would suggest that ammonia nitrogen is not a metabolic requirement for *Thiobacillus denitrificans*, which would be of advantage in the autotrophic denitrification of nitrate containing wastewaters devoid of ammonia. Furthermore, as stated before, it could be demonstrated that orthophosphate could be substituted by polyphosphate.

3.6. Microbial community analysis

Figure 7 shows the scanning electron micrograph of the bacteria clinging to the surface of the sulphur particles. The bacteria are short rods of fairly uniform size and shape with a polar flagellum. Morphologically, they appear to belong to a single species and closely match the description given for *Thiobacillus denitrificans* [26].

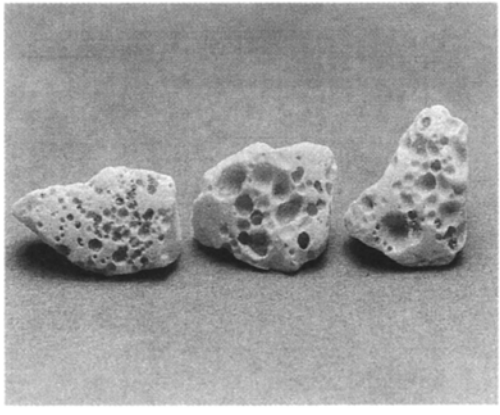

Figure 6. Effect of growth of Thiobacillus denitrificans on the surface of sulphur particles (11.2-16mm)

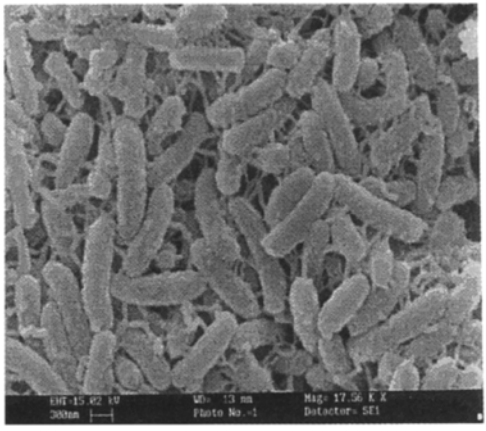

Figure 7. Scanning electron micrograph of bacteria growing on the surface of sulphur particle

3.7. Engineering significance

Autotrophic denitrification of nitrate containing wastewater in sulphur packed beds reactors appears to be an interesting alternative to the heterotrophic denitrification process. It avoids the nagging problem of exactly matching the methanol dosage to varying nitrate concentrations and thus can greatly contribute to more effective and reliable nitrate removal. However, the successful large-scale development of this process still requires further technically demonstrable improvements. Examples of these are: (i) higher volumetric denitrification rates; (ii) deep bed filters or dynamic filters with smaller sulphur particles (1-2 mm diameter) for denitrification of secondary effluents; (iii) application of filter back wash for process control; (iv) development of reliable kinetic prediction model for combined sulphur-limestone packed bed reactors for pH/alkalinity control; (v) control of potential fouling problems using ORP; and (vi) ability to absorb fluctuations in concentrations and shock loads. The apparently low denitrification rates in the autotrophic process when using elemental sulphur may not constitute the limiting factor in the application of this process, since economic aspects, achievable effluent quality, process reliability and stability, ease of operation, etc. also play a very important role in process selection.

4. CONCLUSIONS

Based on the investigations carried out, the following conclusions are drawn:

1. Autotrophic denitrification in sulphur packed bed reactors using *Thiobacillus denitrificans* can effectively remove nitrate from industrial wastewaters and secondary effluent at concentrations up to 800 mg/L NO_3-N. The minimum retention time necessary for complete denitrification depends on sulphur particle size and influent nitrate concentration.
2. Autotrophic denitrification in sulphur packed bed reactors can be described by a half - order reaction rate model for biofilms. The kinetic rate constants for autotrophic denitrification appear to be lower than for heterotrophic denitrification, probably due to slow sulphur dissolution rates.
3. Autotrophic denitrification appears to proceed according to the same metabolic pathway as heterotrophic denitrification, with the rate controlling steps being $NO_2^- \rightarrow N_2O$ and $N_2O \rightarrow N_2$.
4. *Thiobacillus denitrificans* exhibits high salt tolerance, but is sensitive to temperature and pH.
5. The microbial community consists mainly of *Thiobacillus denitrificans* according to microscopic observation.
6. The attached growth of *Thiobacillus denitrificans* leads to the formation of microbially induced holes on the surface of the sulphur particles, contrary to conventional biofilm theory, which assumes uniform biofilm thickness.
7. The observed stoichiometric relationships compare well with theory, but nitrate can substitute for ammonia as a metabolic nitrogen requirement, and polyphosphate can be used instead of orthophosphate.

REFERENCES

1. Batchelor, B., and Lawrence, A.W.(1978). J. Water Poll. Control Fed., 50, 1986-2001.

2. Kruithof, J.C., van Bennekom, C.A., Dierx, H.A., Hijnen, W.A.M, van Paassen, J.A.M., and Schippers, J.C. (1988). Water Supply, 6, 207-217.
3. Gayle, B. P., Sherrard, J. H., and Benoit, R. E. (1989). J. Env. Eng., ASCE, 115, 930-943.
4. Hiscock, K. M., Lloyd, J. W., and Lerner, D. N. (1991). Water Research, 29, 1099-1111.
5. Kapoor, A., and Viraraghavan, T. (1997). J. Env. Eng. ASCE, 123, 371-380.
6. Sant'Anna, F.P.S., Martin, G., and Taha, S. (1996). Rev. Sci. Eaux/J. Water Sci., 9, 333-350.
7. Kim, E.W., and Bae, J.H. (1999). Conference Preprint 7th IAWQ Asia-Pacific Regional Conference October18-20, 1999 Taipei, Taiwan (Ouyang, C.F. *et al.*, eds), Vol. 1, 583-588.
8. Kim, I.S., and Son, J.H. (1999). Conference Preprint 7th IAWQ Asia-Pacific Regional Conference October18-20, 1999 Taipei, Taiwan (Ouyang, C.F. *et al.*, eds), Vol. 1, 107-112.
9. Shan, J., and Zhang, T.C. (1999). Program and Proceedings IAWQ/IWA Conference on Biofilm Systems, October 17-20, 1999 New York.
10. Koenig, A., and Liu, L.H. (1996). Wat. Sci. Tech., 34 (5-6), 469-476.
11. Standard Methods for the Examination of Water and Wastewater (1995). 19th edn., American Public Health Association, Washington DC, USA.
12. Koenig, A., and Liu, L.H. (1997a). Proceedings 8th International Conference on Anaerobic Digestion, May 25-29, 1997, Sendai, Japan, Vol. 1, 299-306.
13. Koenig, A., and Liu, L.H. (1997b). Proceedings Sardinia 97, Sixth International Landfill Symposium S. Margherita di Pula, Cagliari, Italy; 13-17 October 1997 (Christensen, T.H., Cossu, R., and Stegmann, R., eds), Vol II, 283-292.
14. Koenig, A. (1999). Conference Preprint 7th IAWQ Asia-Pacific Regional Conference October18-20, 1999 Taipei, Taiwan (Ouyang, C.F. *et al.*, eds),, Vol. 1, 83-88.
15. Claus, G. and Kutzner, H.J. (1985). Appl. Microbiol. and Biotechnol., 22, 283-288.
16. Kuenen, J.G., Robertson, L.A., and Tuovinen, O.H. (1992). The Prokaryotes Second Edition (eds. Balows, A., Truper, H.G., Dworkin, M., Harder, W., and Schleifer, K.-H.), Vol. III, Chapter 138, 2638-2657, Springer-Verlag, Berlin.
17. Oh, S.-E., Kim, K.-S., Choi, H.-C., and Kim, I.S. (1999). Conference Preprint 7th IAWQ Asia-Pacific Regional Conference October18-20, 1999 Taipei, Taiwan (Ouyang, C.F. *et al.*, eds.), Vol. 1, 173-178.
18. U.S Environmental Protection Agency (1975). Process design manual for nitrogen control. Office of Technology Transfer, Washington, DC.
19. Glass, C., and Silverstein, J. (1998). Water Research, 33, 223-229.
20. Charpentier, J., Martin, G., Wacheux, H., and Gilles, P. (1998). Wat. Sci. Tech., 38(3), 197-208.
21. Flere, J.M., and Zhang, T.C. (1999). J. Env. Eng., ASCE, 125, 721-729.
22. Koenig, A., and Liu, L.H. (1999). submitted for publication.
23. Harremoes, P. (1976). J. Water Pollution Control Federation, 48, 377-388.
24. Henze, M., Harremoes, P., LaCour Jansen, J. and Arvin, E. (1997). Wastewater treatment biological and chemical processes Second edition. Springer, Berlin, 95.
25. Sawyer, C.N., McCarty, P.L., and Parkin, G.F. (1994). Chemistry for environmental engineering Fourth Edition, McGraw-Hill, New York, 305-306.
26. Bergey's Manual of Systematic Bacteriology Vol.3 (1989). (eds. Staley, J.T., Bryant, M.P., Pfennig, N. and Holt, J.G.). Williams & Wilkins, Baltimore.

§4 Waste Sludge Minimization and Materials Recovery

The treatment of waste sludge, which occurs as the result of biological wastewater treatment, is costful and laborious. In this chapter, some topics related with waste sludge reduction and material recovery from wastewater are described.

When we think about the reduction of cost for sludge treatment, waste sludge minimization technologies are very attractive. Different waste sludge minimization metods have been reported so far, for example, ozonation, thermal treatment, and membrane separation. Here, Yamamoto reviews studies on membrane separation activated sludge processes for the minimization of waste sludge reduction.

In waste sludge minimization, organic materials in influent wastewater is mainly converted to carbon dioxide. On the other hand, another way of thinking is to utilize organic materials in influent wastewater by some means. For example, waste sludge has long been used for the production of methane gas as fuel. But here, recent trials on the conversion of organic materials in wastewater into biodegradable plastic are reported by van Loosdrecht *et al.* and Satoh *et al.*. Nakajima *et al.* are working on the conversion of organic materials in wastewater to single cell protein and PHA by using photosynthetic microorganisms.

In all of these studies, it is thought that the detailed understanding on microbial community structure is essential to achieve the best process performances.

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Membrane bioreactor: an advanced wastewater treatment/reclamation technology and its function in excess-sludge minimization

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This paper, first, summarizes performance of membrane bioreactor that is applied to advanced wastewater treatment or reclamation from the viewpoint of a unique hybrid-system. Excess-sludge minimization is then discussed, because one of the most prominent advantages of the membrane bioreactor processes is that the processes are potentially capable of achieving zero-emission in terms of solid waste from them.

1. MEMEBRANE BIOREACTOR –A HYBRID ADVANCED TREATMENT SYSTEM

1.1 Introduction

Water-associated environmental problems people concern about are rapidly expanding in various aspects; e.g., eutrophication and associated odor/taste or endotoxin problems in drinking water, nitrate contamination in groundwater, disinfection byproduct and its precursors, natural and artificial heavy metal pollution, and micropollutants such as carcinogenic and mutagenic compounds, volatile organic compounds, persistent organic pollutants, endocrine disrupters, and so on, in addition to the traditional water pollution caused by organic matters. *Cryptosporidium* oocyst contamination in water supply gives review on hygienically safety for drinking water. Ecological health is also becoming into consideration as well as human health and beneficial uses of waters. It is rather difficult to give a sufficient technological-solution to the arising problems mentioned above by traditional water and wastewater treatment technologies. Needs for advanced treatment, therefore, are increasing in order to especially control micropollutants in water and wastewater.

Furthermore, there is a need for a comprehensive water shed management, emphasizing sustainable water resources development, rational and effective use of water, and promotion of reclaimed water reuse. Direct and indirect wastewater reuse is considered as stable water resources for various beneficial uses of water and for landscape irrigation to restore pleasant water environment. From this viewpoint, advanced treatment must be considered in accordance with various beneficial reuse purposes as well as the aspect of human and environmental health. This is especially important when the treated wastewater is aimed to use for the rehabilitation of urban creak and creation of water environment along it.

Membrane technology is considered as one of the innovative and advanced technologies which rationally and effectively satisfies the above mentioned needs in water and wastewater treatment and reuse.

1.2. Membrane bioreactor as a hybrid system

Membrane technology in combination with biological treatment is reasonably applied to organic wastewaters, a large part of which is biodegradable. The activated sludge process, which is the most typical suspended-growth biological treatment process, normally achieves solid-liquid separation through gravity sedimentation. MSAS (Membrane Separation Activated Sludge Process), a type of membrane bioreactor, is the name given to activated sludge processes in which solid-liquid separation is fulfilled by membrane separation. The membrane separation is usually carried out by means of ultrafiltration (UF) or microfiltration (MF), which do not cause the inhibitive accumulation of salts against microorganisms.

Because membrane separation replaces gravity sedimentation in most cases of recent membrane application to wastewater treatment, it never becomes energy efficient as a unit process of solid liquid separation. Without consideration of needs for advanced or high-performance treatment, it is rather difficult to find space for such a membrane application. We should note that membrane separation is the technology applicable to wastewater reuse as well as treatment.

It is possible, in principle, to achieve any quality of reclaimed wastewater by using only membrane technology because a variety of membrane is available in accordance with the required water quality. However, it is not treatment but just separation, if the concentrate is not being treated. Anyway, wastewater must be treated; therefore, a hybrid membrane system is practically used in combination with biological and/or other physico-chemical treatment processes in most cases.

A hybrid membrane system, MSAS gives a number of advantages through combination with membrane separation from the viewpoint of biological treatment as follows:

1) Since suspended solids are totally eliminated through membrane separation, the settleability of the sludge has absolutely no effect on the quality of the treated water.

2) A long sludge retention time (SRT) can facilitate the proliferation of microorganisms with low growth rate: e.g. nitrifying bacteria[1], higher tropic level maicroorganisms like protozoa and metazoa[2], and so on. It can also minimize sludge production, or sludge mineralization is possible in other words. The role of predators is important in reduction of sludge also[3].

3) The overall performance in biological treatability can be raised, since it is possible to maintain high microbial concentrations in bioreactors while keeping the microorganisms dispersed in a relatively small floc-size [4].

4) High concentrations of the sludges create a favorable environment for "endogenous" denitrification, thereby ensuring the efficient removal of nitrogen[5,6].

5) Treatment efficiency is also improved in the sense that it is possible to retain undecomposed polymer substances until their degradation[7].

6) Membrane itself and/or dynamic membrane effect mainly due to cake/gel formation can achieve a high enough removal of bacteria and viruses, although membranes are not complete barriers for them [8,9].

From the perspective of membrane separation, the possible advantages of the combination with biological treatment include the followings.

1) Dissolved organic substances with low molecular weights, which cannot be eliminated by membrane separation alone, can be taken up, broken down and gasified by microorganisms or converted into polymers as constituents of bacterial cells, thereby raising the quality of treated water.

2) Polymer substances retained by the membranes can be broken down if they are still biodegradable, which means that there will be no endless accumulation of the substances

within the treatment process. This, however, requires the balance between the production and degradation rates. A high organic loading might give a higher production rate of intermediate polymer metabolites than that of the degradation, resulting in their accumulation. The accumulation of intermediate metabolites may decrease the microbial activities in the reactor [10].

Table 1 summarizes the expected performance of MBR for wastewater treatment.

As summarized above, the membrane bioreactor gains multiple merit by its hybrid nature. Furthermore, we can expect that the suspended-solid free effluent gives another advantage to a post-treatment (such as activated carbon adsorption, UV irradiation and so on), if it is considered. The function of membrane separation in the hybrid system is, anyway, more than solid-liquid separation.

1.3. Performance of MSAS

As mentioned above, MSAS normally leads to operation with high concentration of activated sludges, which can easily absorb shock loadings and give very stable treatability. The performance of MSAS may be affected by irreversible changes in the membranes themselves, or by the fouling of membrane interiors or surfaces. The permeability tends to mainly due to the formation of cake/gel layers. The actual rejection of ultrafiltration and microfiltration membranes is often similar [7] since the cake/gel layers formed control the separation.

Table 1
Expected performance of MBR for wastewater treatment

	Expected Performance
Suspended Solids	Complete removal No influence of sludge settleability on effluent quality Removal of particle-bound micropollutants
Virus, Bacteria, Protozoa	Reliable removal by size exclusion, retention by dynamic membrane, a high removal along with SS retention
Nitrogen	Stable nitrification due to high retention of nitrifying bacteria Low temperature nitrification is attained A high effectiveness factor in terms of nitrification due to relatively small size floc Endogenous denitrification is highly expected due to high concentration of biomass
Sludge stabilization	Minimize excess sludge production due to long SRT Sludge treatment is possible together with wastewater treatment Use of higher tropic level of organism is expected to control sludge
Degradation of hazardous substances	Selective growth of specific microorganisms is expected for hardly degradable hazardous substances Almost pure culture system is easily operated

When sludge concentration exceeds a certain limit, the permeation flux rapidly decline due to a dramatic rise in viscosity of the sludge mixture. The limit for the filtration of the activated sludge concentration is 30,000-40,000 mg/L [11,12]. Most practical applications are operated at less than 20,000mg/L. A guideline value of 10,000 –20,000 mg/L is recommended for submerged MSAS for on-site domestic wastewater treatment [13]. The correlation between sludge concentration and permeate flux is not clear when the sludge concentration is below the threshold one. The colloidal or soluble substances, which cannot be measured in terms of sludge concentration within the sludge mixture, would become more important.

Unlike the filtration of pure water, there is no guarantee that there will be linear relationship between operating pressure and permeate flux. An increase in pressure sometimes causes a decline in the permeate flux, and it is probably due to the compaction of the cake layer. Control of the cake layer plays an important role in the reduction of fouling, since the cake layer on the surface of the membrane dominates the filtration. One well-known approach is to improve the scouring effect by increasing the tangential flow velocity, but this method has the drawback of increasing energy consumption. Critical flux concept is another approach to control the fouling [14,15,16]. The critical flux is ideally defined as the flux below which no fouling takes place. It is also important to reduce compaction of the cake layer. Low-pressure filtration and intermittent filtration, therefore, appear to offer an effective means of achieving this.

1.4. Submerged MSAS for domestic and industrial wastewater treatment and reclamation

A new generation of MSAS is the submerged type where membrane modules are directly immersed in an aeration tank. This aims to significantly reduce the energy consumption by eliminating a big circulation pump typically installed in a conventional MSAS [4].

For submerged MSAS, hollow-fiber and plate and flame type modules in microfiltration range have been used in most cases. A number of applications is increasing in industrial wastewater treatment, on-site domestic wastewater treatment (Jokaso), municipal wastewater treatment, night soil treatment and so on [17,18,19, 20,21,22,23].

Aeration is supplied underneath the submerged modules, generating upflow inside the module and preventing it from fatal fouling as well as oxygen supply for biological reaction. In the case of hollow fiber, the aeration is also utilized to make oscillatory movement of the fibers, which is considered effective for washing fibers and controlling of inter-fiber clogging. The operation of submerged MSAS is characterized as relatively low pressure and low flux operation, which is supported by a critical flux theory, in order to prolong the life of membrane and to reduce the maintenance requirement that is especially important for small-scale treatment plant operation. Intermittent filtration by a suction pump is practically adopted in many submerged MSAS plants. A gravity head filtration is also applicable [13].

It would be easier to apply a submerged MSAS to retrofit of an outdated wastewater treatment plant, where aeration tanks and sedimentation tanks could be utilized and improved by only adding membrane modules in the tanks. The retrofit of municipal wastewater treatment plant has been implemented by a submerged hollow fiber module system in Canada [22].

It is noted that a compact submerged MSAS unit is considered promising in retrofit and improvement of Japanese traditional household wastewater treatment system (old-type Jokaso), which treats only toilet flush-water and untreated graywater has caused water pollution problems in many water bodies in Japan. Improvement of the system achieving

graywater-included treatment is urgently required, but space for additional treatment is rather limited in such small land availability for typical individually owned house, to which a compact submerged MSAS would help a lot. Furthermore, a high quality of membrane effluent is potentially reused or infiltrated in an individual house-scale.

1.5. Membrane bioreactor perspective

Historically, UF membrane bioreactor first appeared in application to wastewater treatment, then MF membrane bioreactor followed. As the main function of the membrane is to separate solid particles from the treated wastewater, and UF and MF filtrates gave practically same effluent water quality, there is no special reason to use tight UF as a solid-liquid separation device of the bioreactor. Therefore, there will be a trend of the use of MF or lager pore-size UF membrane, instead using tight UF membrane, in order to get a high filterability.

On the other hand, it is pointed out that the demands for advanced treatment in terms of further removal of dissolved and small molecular-weight organic matters will certainly increase in near future, then neither UF nor MF is not sufficient any more. A post-treatment might be required to the membrane bioreactor effluent for that purpose. Nanofiltration (NF) is one of the candidates of the post-treatment. It is also worthwhile to investigate a NF membrane bioreactor where a NF membrane with a low salt-retention but with a high retention in terms of organic matters must be considered.

2. EXCESS-SLUDGE MINIMIZATION IN MEMBRANE BIOREACTOR

As already mentioned above, a long SRT with which a typical membrane bioreactor is operated can minimize excess-sludge production. This is not special at all; any process that achieves long SRT should give similar results. It is noted that, however, a membrane bioreactor easily satisfies a long SRT condition even at a high organic loading without deteriorating the effluent quality nor losing the sludge simply because of membrane separation, by which a high sludge concentration is always maintained. This seems very difficult to be obtained by conventional activated sludge processes utilizing gravity settling for solid-liquid separation.

2.1 Theoretical aspects (fundamentals)

Assuming growth yield and endogenous decay coefficient are constant, a traditional steady-state kinetics gives an estimation of observed yield:

$$Yob = \frac{\delta X}{\delta S} = \frac{Y}{1 + b \cdot SRT} \qquad (1)$$

where, Yobs: observed yield that is defined by $\delta X / \delta S$

$\delta X / \delta S$: the ratio of excess biological-mass (sludge) removed to organic substrate removed

SRT: sludge retention time, d

b: overall endogenous decay coefficient, d^{-1}

Y: growth yield of heterotrophic microorganisms

The value of Y in membrane bioreactors has been reported about 0.6 [12,24]. The typical value of b has been reported about 0.05 d^{-1} [3]. Using these values, we can easily draw the relationship between Yobs and SRT, as shown in Figure 1.

Although the above relation is too simple to cover the complicated phenomena that must be actually happen in the membrane bioreactor, we can clearly understand that we need a very long SRT probably of greater than 500 days, by which we can achieve nearly zero excess-sludge production.

2.2 Enhancement of sludge mineralization

Considering the case of a very long SRT and assuming 100% removal of substrate, we can obtain an approximate equation:

$$F/M = b/Y \quad \text{or} \quad X = YL/b = L/(F/M) \tag{2}$$

Where, F/M: food-microorganism ratio, kg/(kg d)

X: Biological mass concentration, kg/m^3

L: Volumetric organic loading, $kg/(m^3\ d)$

Typically, the order of magnitude of b is one tenth of that of Y, F/M must be in the order of magnitude of 0.1 d^{-1}. The reported values of F/M obtained in membrane bioreactors vary from 0.05 to 0.24 d^{-1} [5,11,12,24,25]. Suppose L is given as 2 $kg/(m^3\ d)$, X varies from 8300 mg/L to 40,000 mg/L in accordance with the variation of F/M. As already mentioned, the MLSS is recommended not exceed 20,000 mg/L [13]. Therefore, it is very important to control the F/M, in other words, b/Y. The growth yield, Y, must not be varied much, then overall endogenous decay coefficient, b, which is related to the sludge mineralization rate, is primarily responsible to a significant change in F/M. The value of b must depend on various

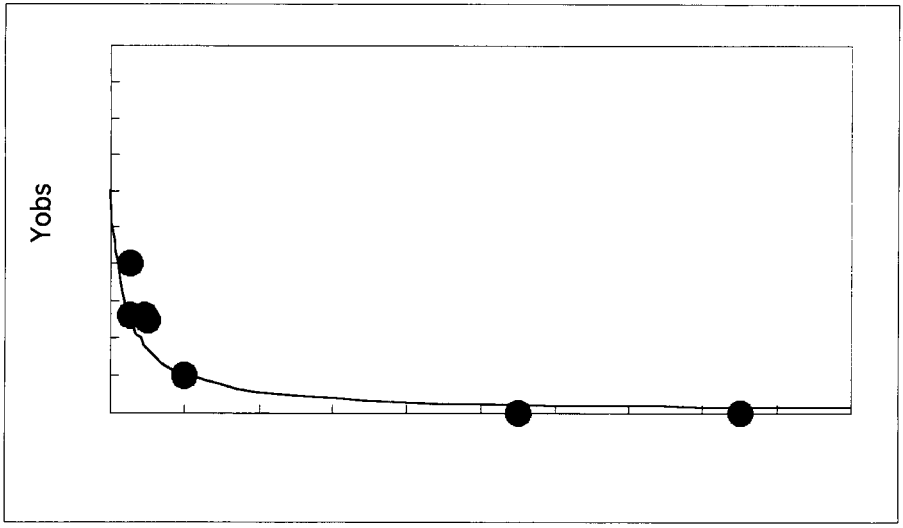

Figure 1. Illustrative example of relationship between Yobs and SRT. The curve line is drawn by using Eq.(1) and the solid circles are the experimental data obtained in membrane bioreactor, which are adopted from the references[5,12,21].

factors, such as dissolved oxygen concentration, pH, temperature, and others, or the stresses caused by change in environmental conditions. Moreover, the spices structure of microbial ecosystem in the membrane bioreactor must also affect b.

Zhang[3] pointed out the role of predators in the sludge mineralization and showed a simulated result that 40% reduction of X could be achieved by the contribution of predators, by formulating and using an ecological system model of prey-predator relationships among bacteria, protozoa and metazoa.

However, the predators sometimes fluctuate a lot [2] or even the case was reported that no predator was observed [25]. Luxmy et al.[26] suggested that the fluctuation of predator population was affected by the availability of dispersed bacteria in the bioreactor(Figure2). It is still unclear which condition gives a stable growth of predators that contributes a significant sludge mineralization.

There is still a lot of space for detailed investigation on that.

3. SUMMARY

Membrane technology in combination with biological treatment is reasonably applied to organic wastewaters, a large part of which is biodegradable. It is possible, in principle, to achieve any quality of reclaimed wastewater by using only membrane technology because a variety of membrane is available in accordance with the required water quality. However, it is not treatment but just separation, if the concentrate is not being treated. Anyway, wastewater must be treated; therefore, a hybrid membrane system is practically used in combination with biological and/or other physico-chemical treatment processes in most cases. The membrane bioreactor gains multiple merit by its hybrid nature. Furthermore, we can expect that the suspended-solid free effluent gives another advantage to a post-treatment (such as activated carbon adsorption, UV irradiation and so on), if it is considered. The function of membrane separation in the hybrid system is, anyway, more than solid-liquid separation.

A long SRT with which a typical membrane bioreactor is operated can minimize excess-sludge production. This is not special at all; any process that achieves long SRT should give similar results. It is noted that, however, a membrane bioreactor easily satisfies a long SRT

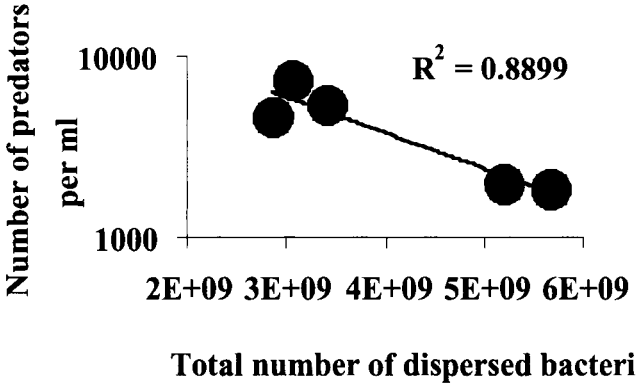

Figure 2. Correlation between total predator number with total dispersed bacterial population[2]

condition even at a high organic loading without deteriorating the effluent quality nor losing the sludge simply because of membrane separation, by which a high sludge concentration is always maintained. This seems very difficult to be obtained by conventional activated sludge processes utilizing gravity settling for solid-liquid separation. Although the fundamental relation discussed here is too simple to cover the complicated phenomena that must be actually happen in the membrane bioreactor, we can clearly understand that we need a very long SRT probably of greater than 500 days, by which we can achieve nearly zero excess-sludge production. It is very important to control the F/M, in other words, b/Y. The growth yield, Y, must not be varied much, then overall endogenous decay coefficient, b, which is related to the sludge mineralization rate, is primarily responsible to a significant change in F/M. The value of b must depend on various factors, such as dissolved oxygen concentration, pH, temperature, and others, or the stresses caused by change in environmental conditions. Moreover, the spices structure of microbial ecosystem in the membrane bioreactor must also affect b.

REFERENCES

1. B.S.Luxmy, F.Nakajima,and K.Yamamoto, Analysis of bacteria community in membrane separation bioreactors by fluorescent *in situ* hybridization (FISH) and denaturing gradient gel electrophoresis (DGGE) techniques, Proc. Int. Specialized Conf. on Membrane Technology in Environmental Management, Tokyo, November (1999)302.
2. B.Zhang and K.Yamamoto, Seasonal changes of microbial population and activities in building wastewater reuse system using a membrane separation activated sludge process, Water Sci. & Tech., 34, 5/6(1996)295.
3. Boran Zhang, A study on microbial activities and the role of predators in membrane separation activated sludge process, PhD Thesis, The University of Tokyo (1997).
4. B.Zhang,K.Yamamoto,S.Ohgaki,and N.Kamiko, Floc size distribution and bacteria activities in membrane separation activated sludge process for small-scale wastewater treatment and reclamation, Water Sci. & Tech., 35, 6(1997)37.
5. C. Chiemchaisri, Y.K. Wong, T.Urase and K. Yamamoto, Organic stabilization and nitrogen removal in membrane separation bioreactor for domestic wastewater treatment, Water Sci. & Tech., 25, 10(1992)231.
6. C. Chiemchaisri and K. Yamamoto,Biological nitrogen removal under low temperature in a membrane separation bioreactor, Water Sci. & Tech., 28, 10(1993)325.
7. Y. Fuchigami, K. Yamamoto, K. Asami and T. Matsuo, On the activated sludge process combined with membrane separation, Proc. of Environ. & Sanitary Eng. Res., 23(1987)239 (in Japanese).
8. T. Urase, K. Yamamoto, S. Ohgaki, Effect of pore structure of membranes and module configulation on virus retention, J. Membrane Science, 115 (1996) 21.
9. H.Gemunu, K.Yamamoto, and T.Urase, Mechanism of bacterial and viral transport through microfiltration membranes, Water Sci. & Tech., 38, 4/5(1998)489.
10. K. Nishimura, Y. Ogata, M.Arano, S. Kawamura and Y. Magara, Proc. of Annual Meeting of JSWE (1994)(in Japanese).
11. K.Yamamoto, M.Hiasa, M.Mahmood and T.Matsuo, Direct solid-liquid separation using hollow fiber membrane in an activated sludge aeration tank, Water Sci. & Tech., 21,

4/5(1989)43.
12. K. Yamamoto and K.M.Win, Tannery wastewater treatment using a sequencing batch membrane reactor, Water Sci. & Tech., 23,7/9(1991) 1639.
13. Building Research Insitute, Ministry of Construction "Design and management guidelines for the development of advanced on-site domestic wastewater treatment facilities using membrane filtration", J. Water & Wastewater, 40(1998)241.
14. Dae-Young Kwon, Experimental investigation on critical flux in cross-flow microfiltration, PhD Thesis, University of Tech-nology, Sydney (1998)
15. A.G.Fane, P.Beaston and H.Li, Membrane fouling and its control in environmental applications, Proc. Int. Specialized Conf. on Membrane Technology in Environmental Management, Tokyo, November (1999)12.
16. S.Vigneswaran, D.Y.Kwon, H.H.Ngo and J.Y.Hu, Improvement of microfiltration performance in water treatment: Is critical flux, a viable solution?, Proc. Int. Specialized Conf. on Membrane Technology in Environmental Management, Tokyo, November (1999)20.
17. N. Yamamoto, Proc. 29th Annu. Meeting on Japan Sewage Works Association (1992) (in Japanese)
18. O. Futamura, Seminar Text on latest trend in development of MSAS, Technical Information Center (1995) (in Japanese)
19. Y. Narukami, Seminar Text on latest trend in development of MSAS,Technical Information Center (1995) (in Japanese)
20. H. Ishida, S. Izumi and M. Moro, Proc. of 2nd Annu. Meeting on Japan Society of Waste management Experts (1992) (in Japanese)
21. R.Ben Aim, Membrane bioreactors with submerged hollow fiber membranes: from lab-scale to industrial scale, Distributed documents, Int. Specialized Conf. on Membrane Technology in Environmental Management, Tokyo, November (1999).
22. P. Cote and D.Thompson, Wastewater treatment using membranes: the north American experience, Proc. Int. Specialized Conf. on Membrane Technology in Environmental Management, Tokyo, November (1999)46.
23. H.Ohmori, T.Yahashi, Y.Furukawa, K.Kawamura, and Y.Yamamoto, Treatment performance for newly developed Johkasous with membrane separation –on site system for domestic sewage treatment in Japan, Proc. Int. Specialized Conf. on Membrane Technology in Environmental Management, Tokyo, November (1999)38.
24. J.Wagner and K.H.Rosenwinkel, Sludge production in membrane bioreactors under different conditions, Proc. Int. Specialized Conf. on Membrane Technology in Environmental Management, Tokyo, November (1999)294.
25. S.Rosenberger, M.Kraume, and U.Szewzyk, Operation of different membrane bioreactors: experimental results and physiological state of the microorganisms, Proc. Int. Specialized Conf. on Membrane Technology in Environmental Management, Tokyo, November (1999)310.
26. B.S.Luxmy, F.Nakajima, and K.Yamamoto, Predator grazing effect on bacterial size distribution and floc size variation in membrane separation activated sludge, Proc. of Asian WaterQual'99, Vol.1, Taipei, October (1999)728.

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Poly-β-hydroxyalkanoate metabolism in activated sludge

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In this contribution an overview of the conversion processes of the storage polymer poly-β-hydroxyalkanoate is given. This polymer plays an essential role as intermediate in the oxidation of soluble organic substrates in activated sludge cultures. The stoichiometry can well be described based on a metabolic model. For the process kinetics there is some indications on a possible kinetic description, however more research is needed before a reliable description can be offered.

1. INTRODUCTION

Generally microbial conversion processes in activated sludge are described by uptake of substrate followed by conversion of the substrate to biomass. The biomass itself is subsequently used for endogenous respiration or decay. This is a highly simplified view, mainly based on the fact that general microbial research is devoted to batch cultures or continuous cultures. In nature these conditions probably hardly exist. Periods of substrate availability are interchanged by periods of 'starvation'. These conditions can be induced by e.g. tidal flows, the light-dark cycles, or the variable influent composition and flow in a wastewater treatment plant.

Wastewater treatment processes are widely used microbial ecosystems subjected to so-called feast-famine regimes. For sequencing batch processes this is evident, but also continuous operated systems are subject to feast-famine regimes. This is partly caused by the non-continuous feeding to the process but also results from the fact that treatment processes very seldom consist of well-mixed reactors. Many processes contain combinations of tanks or have a plug flow character, resulting in only a small fraction of the sludge being in direct contact with the influent-COD.

The traditional view of growth-starvation processes, as expressed in e.g. the general used activated sludge model no 1 [1], is however too simplistic. Microorganisms tend to respond to dynamic feeding patterns in much the same manner as higher organisms. During feast periods (feeding periods) the food is partly accumulating as an intracellular storage polymer. When there is no external supply of carbon or energy source (famine period) the stored polymer is used for growth and cell maintenance processes [2]. These storage polymers can be polyhydroxyalkanoates (PHA), lipids, or glycogen [3]. In activated sludge processes PHA can

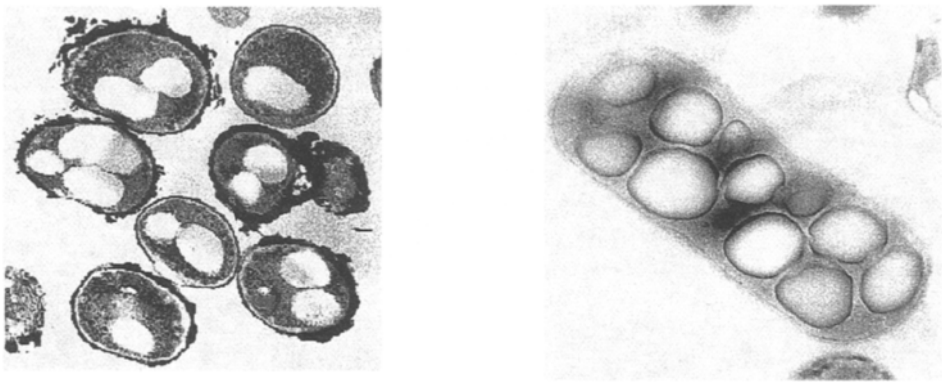

Figure 1. Examples of PHB accumulated in activated sludge.

be considered the dominant storage polymer (Figure 1). Only when significant amounts of glucose are present also glycogen will be accumulated [4,5]. Due to rapid glucose conversion in sewers this will however be rarely the case.

In wastewater treatment processes formation of storage polymers is involved in several important processes:

- *Phosphate accumulating organisms (PAO).* This is probably the most widely recognized microbial process where storage polymers (PHB, Glycogen and polyphosphate) play an essential role. The whole competitive advantage for these organisms is based on their capacity to utilize the energy stored as poly-P to store exogenous substrate as PHB when there is no external electron acceptor (oxygen or nitrate) available for energy generation [6].
- *Glycogen accumulating organisms (GAO).* These bacteria have recently been recognized since they compete with the PAO [7]. Effectively these organisms rely on substrates which can be fermented (e.g. glucose), while they store the fermentation products inside the cell rather then excreting them. These organisms can also utilize internal stored glycogen for fermentation to PHB. The energy released in the glycolysis process is subsequently used to accumulate fermentation products (e.g. acetate) as PHB. Both PAO and GAO proliferate in systems where regularly substrate is present, while at the same time electron acceptors are absent.
- *Bulking sludge control.* Traditionally competition between floc-forming and filamentous organisms is based on the assumption that filamentous bacteria proliferate at low substrate concentration in the reactor. Plug flow reactors (selectors) can prevent the proliferation of filamentous bacteria. Already in the original papers of Chudoba *et al.* (1973) [8] it is clear that a large fraction of the substrate is stored inside the cell after which it is consumed during the remainder of the process. This has been studied by Van den Eijnde *et al.* (1984) [9].
- *Return Activated Sludge Processes* (RAS). Several authors (e.g. Ulrich and Smith 1951 [10]) have observed that with a relative short hydraulic retention time the COD is

already removed from the liquid phase, however a long 'endogenous' phase is needed in order to maintain in the long run a good treatment capacity. It is suggested in order to save space to separate the sludge and effluent at the moment the soluble COD is taken up by the sludge, and provides an extra aeration tank for the 'endogenous' respiration in the return sludge line. In this tank not only particulate substrates are hydrolyzed, but also the internal stored substrates are consumed and used for sludge growth.

- *General COD conversion.* It has become evident that a large fraction of the soluble COD is first converted to storage polymers, after which it is used for growth [11]. In this manner the organisms more or less balance their growth rate. There is no need to induce all the growth-related enzymes at maximum level, in order to compete for substrate. A fast uptake rate of substrate is essential for this competition, not a fast growth rate. Since the number of enzymes involved in substrate uptake and storage is very limited, this is more efficient. Bacteria without the capacity to store substrates will rapidly be outcompeted in a feast-famine regime. Substrate storage is especially relevant for denitrification processes. Storage of substrate needs less energy (electron acceptor) then growth processes. If substrate storage occurs this leads to an inefficient use of the substrate in denitrification processes.
- *Recovery of organic carbon.* PHB is a biopolymer with almost the same characteristics as polypropylene. PHB is already produced industrially. The characteristic of the activated sludge to form PHB opens the opportunity to recover organic carbon from wastewater in a reusable form.

Recognition of the importance of storage polymers has led to the proposal of ASM3 [11] by the IAWQ task group as a replacement of ASM1. This is done because the use of ASM1 led to some strange observations; e.g. when acetate is dosed in a standard respirometric test for sludge characterization, very high yield coefficients are observed (up to 0.8 g/g). Inclusion of storage polymers in the model description also allowed the growth-decay model with a model based on endogenous respiration/maintenance. The latter has also extra advantages for parameter estimation procedures.

The kinetics of storage polymer formation or consumption have, however, hardly been studied. Traditional microbiological research orients at the formation of polymers under growth limiting conditions. In activated sludge processes substrate is taken-up and used for growth and storage at the same time. Simultaneous growth and storage, and growth on internal storage products is not studied in detail at all. This means that the wastewater engineer has to propose and assume kinetic rate expressions. It is clear that conventional Michaelis Menten kinetics or Monod kinetics as developed for growth on soluble substrates do not hold under feast-famine conditions. However for the time being in ASM3 a choice for these much used traditional kinetic equations has been made.

2. GENERAL MICROBIAL RESPONSE TO NON-STEADY STATE CONDITIONS

The effect of non-steady state conditions can be best studied by applying a sudden change in a steady-state chemostat culture of a single organism [12]. In Figure 2 the effect of a sudden pulse of substrate to a steady-state is shown. In steady state a low concentration of PHB is already present in the cells. It is not clear whether this PHB is present as storage polymer or

has a direct metabolic function. As soon as the organisms are confronted with an increased substrate concentration in the bulk liquid, they respond in accordance to the general kinetics for substrate uptake. The specific substrate uptake rate increases momentarily to almost the maximal rate. From the linear decrease in substrate concentration it appears that in time this uptake rate remains constant. This increased substrate uptake rate is not compensated by a momentarily increase in growth rate (as evaluated from the ammonium uptake rate and the C- and COD-balance of the growth system). The unbalance between substrate uptake and growth rate is compensate by storing the surplus uptake as PHB. In time the growth rate increases towards the maximal growth rate of the organisms, with a concomitant decrease of the PHB formation rate. This increase of growth rate seems to be dependent on the previous growth rate in the system, with a slower increase if the organisms grew at a lower growth rate [12]. When the substrate is depleted, the organisms start to consume the internal stored PHB. This is used for growth but at a lower rate. This indicates that the PHB degradation rate is the growth rate determining process, rather then that the consumption of PHB is driven by the growth rate of the organisms.

3. METABOLIC MODELING OF MICROBIAL STORAGE PROCESSES

Use of a metabolically based model for complex microbial processes such as formation and consumption of storage polymers has as advantage that the stoichiometric parameters are more consistently described. The macroscopic yield coefficients for growth on substrate (here acetate), polyhydroxybutyrate (PHB) formation and growth on PHB are not independent. From a metabolic model it will become evident that in fact these three parameters all relate to one parameter which describes the efficiency of the energy generation in a cell (the ATP to

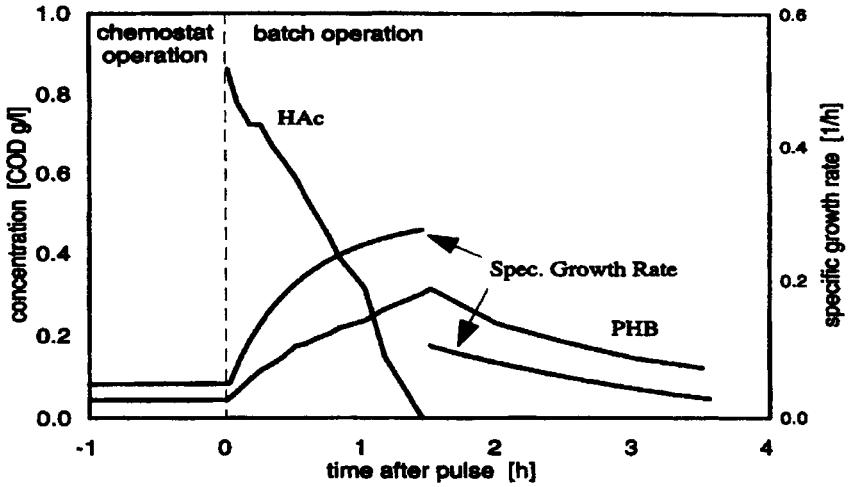

Figure 2. Change in a *P. pantotrophus* culture upon change from chemostat to batch mode of growth and supply of a pulse of substrate.

oxygen or P/O ratio). The principle of metabolic modelling is a description of the metabolism as a set of known basic cell internal metabolic reactions [13]. These reactions are linked to each other by their use of Acetyl-CoA, NADH or ATP. Since these electron and energy carriers in the cell have a more or less constant internal cellular concentration they can be used in a steady state balance. Using this approach leads to an expression similar to the Herbert-Pirt relation for microbial growth but with metabolic yields instead of macroscopic yields as parameters. Previously we described in a similar manner the conversions by polyphosphate accumulating organisms [14]

The metabolic model was formulated by Van Aalst-van Leeuwen *et al.* (1997) [12] and Beun *et al.* (2000) [15,16]. In the description below the elemental biomass composition used was experimentally derived as $CH_{1.87}O_{0.66}N_{0.17}$.

Reaction 1: Synthesis of Acetyl-CoA from acetate

Acetate is taken up and converted into the central metabolite acetyl-CoA. For this process 2 ATP per mole of acetate is required.

$$0.5\ CH_3COOH - 0.5\ HSCoA - 1\ ATP + 0.5\ Acetyl\text{-}CoA + 0.5\ H_2O = 0$$

Reaction 2: Anabolism

The anabolism is described as formation of biomass monomers from acetyl-CoA. In this process ATP is consumed and CO_2 is formed by decarboxylation processes. These processes have been studied in detail by Stouthamer (1973) [17]. Cell maintenance processes are incorporated by m_{ATP} which is growth rate (μ) dependent

$$0.63\ Acetyl\text{-}CoA - 0.17\ NH_3 - 0.66\ ATP - (1.5+m_{ATP}/\mu)\ ATP + CH_{1.87}O_{0.66}N_{0.17} + 0.26\ CO_2 - 0.55\ H_2O + 0.76\ NADH_2 = 0$$

Reaction 3: Catabolism

The catabolism (oxydation to CO_2) of acetyl-CoA can be written as [18]:

$$-0.25\ Acetyl\text{-}CoA - 0.75\ H_2O + 0.5\ CO_2 + 1\ NADH_2 = 0$$

Reaction 4: Electron transport phosphorylation

This reaction describes the oxidation of $NADH_2$ whereby ATP is produced. The δ (or the P/O ratio) describes the energetic efficiency of the reaction [13] and is considered to be independent of the growth rate.

$$-NADH_2 - 0.5\,O_2 + H_2O + \delta\ ATP = 0$$

Reaction 5: Synthesis of PHB from Acetyl-CoA

$$-0.5\ Acetyl\text{-}CoA - 0.25\ NADH_2 + 0.25\ C_4H_6O_2 = 0$$

Reaction 6: Synthesis of Acetyl-CoA from PHB

$$-0.25\ C_4H_6O_2 - 0.25\ ATP + 0.5\ Acetyl\text{-}CoA + 0.25\ NADH_2$$

For the feast phase reaction 1-5 apply, for the famine phase reaction 2,3,4,and 6 apply. Now for each phase a balance for ATP, NADH, and Ac-CoA can be defined, where the net conversion of these compounds can be assumed equal to zero. Substitution of these balances lead to:

(i) a linear equation for the feast period, describing acetate uptake (r_S), biomass growth (r_X), PHB production (r_P) and maintenance (m_S):

$$(-r_s) = \frac{1}{Y_{sx}^{max}}(r_x) + \frac{1}{Y_{sp}^{max}}(r_p) + m_s C_x$$

(ii) a linear equation for the famine period, describing PHB consumption, biomass growth and maintenance:

$$(-r_p) = \frac{1}{Y_{px}^{max}}(r_x) + m_p C_x$$

with the following expressions for the yield factors and the maintenance coefficients:

$$Y_{sx}^{max} = \frac{4\delta - 2}{4.04\delta + 4.32} \qquad m_s = \frac{m_{ATP}}{2\delta - 1}$$

$$Y_{sp}^{max} = \frac{4\delta - 2}{4.5\delta} \qquad m_p = \frac{m_{ATP}}{2.25\delta - 0.25}$$

$$Y_{px}^{max} = \frac{4.5\delta - 0.50}{4.04\delta + 4.32}$$

From above equations it can be seen that five macroscopic yield and maintenance coefficients are needed in a conventional description of PHB formation and consumption as in ASM3. By using the known information of bacterial metabolism these 5 parameters can be related to only 2 true independent variables. If also the denitrification processes would have to be included this would in a conventional description lead to 5 extra parameters, whereas in a metabolic description only an extra δ for the ATP per 2 electrons donated to nitrate is needed. The latter can in principle be related to the difference in Gibbs free energy between oxygen and nitrate as electron donor [19]. It is worthwhile to note that despite a metabolic description seems at first sight to be more complex than a black box description, the end result is less complex (less parameters required). Recognizing the metabolic relation between the macroscopic yield coefficients is also useful when parameters have to be estimated. In principle the values of the different macroscopic yield coefficients cannot be changed independent. Without a metabolic relation this is however often the practice.

4. PHB ACCUMULATION AND CONVERSION IN MIXED CULTURES

A representative illustration of the conversions in a cyclically operated batch reactor with a mixed culture growing on acetate is given in Figure 3. Directly upon dosing the influent to the reactor acetate is consumed and used for storage and growth processes. After this rapid uptake the accumulated PHB is used for growth and maintenance processes. During the feast period, the growth is faster than during the famine period. It appeared that the biomass specific acetate uptake rate depended on the SRT at which the culture was growing [15,16]. When the

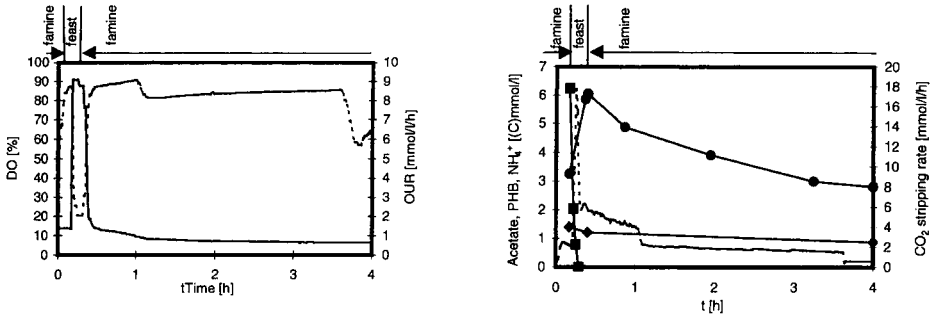

Figure 3. Illustration of changes in conversion rates and concentrations during one cycle in the acetate fed SBR process at a SRT of 3.8 days, measured in several cycles. (a) (—) oxygen uptake rate (OUR) and (---) dissolved oxygen (DO). (b) (■) Acetate; (◆) NH_4^+; (●) PHB; (---) CO_2 stripping rate.

influent addition is more spread out over the cycle the same pattern occurs, although the conversion rates in the feast phase are limited by the substrate addition rate.

When estimating the metabolic yield coefficients (P/O ratio and the m_{ATP}) from different cultures (aerobic and anoxic PHB formation, and growth on acetate or glucose, or biological P-removal), these values differ slightly [5,15,16]. However part of this variation is caused by the fact that both coefficients are highly interdependent and can difficultly be estimated independent. The combination of the two coefficients gave in each case non-significant different result for the macroscopic yield coefficients. This might indicate that the metabolic yield coefficients could well be used as general yield coefficients for all organisms in a mixed culture.

The values for the metabolic yield coefficients found are 0.48 (biomass formation on acetate), 0.67 (PHB formation on acetate) and 0.68 (biomass formation on PHA) C-mol/C-mol [15,16]. When comparing growth on the primary substrate to accumulation of PHB and subsequent growth on PHB, the net yield is approx. 7% decreased due to the conversion via a storage polymer. Apparently the storage mechanism of PHB is energetically efficient. For a wastewater treatment plant this means that stimulating the PHB pathway for growth will only marginally decrease sludge production. For the micro-organisms involved it means that despite a slightly lower growth yield, they can effectively compete with non-PHB storing bacteria. The substrate storage lets the organisms easily survive the periods without feeding, and the fast uptake of substrate in the feast period does not require full induction of all growth related enzymes. The latter could maybe easily balance the slight energy loss due to the storage metabolism.

5. KINETICS OF PHB CONVERSION

Polymer formation

Experimentally the maximal substrate uptake rate appears to depend on the average growth rate (or the solids retention time, SRT) at which the culture is growing [15,16]. The

experimental results of several different mixed culture experiments indicate that the ratio between the biomass specific PHB storage rate and substrate uptake rate might be more or less constant (0.4-0.6 C-mol/C-mol). This ratio could even be applied in cases where the substrate was dosed such that the uptake rate was well below the maximal uptake rate [20]. If this constant ratio is found over a wider range of experimental conditions, it could be very useful for modeling of storage processes in an activated sludge model. Due to metabolic constraints then only one rate coefficient needs to be evaluated in order to describe the feast phase. At this moment there is no basic indication that this ratio should be constant, it could well be coincidental.

Polymer consumption

The specific PHB consumption rate as a function of the PHB content of the biomass seems to be independent of the average growth rate of the culture, indicating that PHB conversion is the rate limiting process and not the growth of the cells. However the average PHB level in the cells varies for cultures at different SRT's [15,16,20]. Clearly the PHB content is a reflection of the average growth rate of the cells (more or less 1/SRT), just as the soluble substrate concentration reflects the growth rate in a chemostat culture. There is, as yet, no good kinetic rate expression for consumption of internal storage polymers by microbial cells. Assuming a Monod-type of relation seems to be rather simplistic. Most of the results obtained until now indicate a first order rate equation in the PHB content of the cells to be appropriate for the conversion of PHB. Other rate orders have been proposed [18], but upon analyzing in detail they usually do not differ significantly from a first order expression, within the experimental range.

Effect of temperature

Krishna *et al.* (1999) [20] have studied the influence of temperature on the accumulation of PHB in SBR cultures fed with acetate. In these cultures the acetate dosage rate was rate limiting, ie the acetate concentration was always near zero during the feast phase. It was shown that in these cases the PHB formation rate decreased as a function of temperature. This was probably the result of an increased anabolic rate at higher temperatures, indicating that the PHB formation process would be the resultant of the Acetyl-CA formation from the substrate and Acetyl-CoA consumption of the anabolic processes. Also other aspects such as the biochemical control of the process might point in that direction. Based on this Krishna *et al.* (1999) [20] postulated the scheme in Figure 4 for the metabolic control of the PHB formation process and growth related processes during the feast phase.

6. CONCLUSIONS

It is now generally recognized that soluble substrate conversion in activated sludge cultures is associated to storage polymer metabolism. For a proper description of these more complex microbial conversions it is shown that a metabolic based model leads to a more consistent description with less parameters then a conventional black box description for the process stoichiometry. For the reaction kinetics initial data and proposals for reaction rate equation are available. However much more detailed analyses of kinetic experiments is needed. This

should be combined with a more detailed mechanistical description of the processes at the enzyme level.

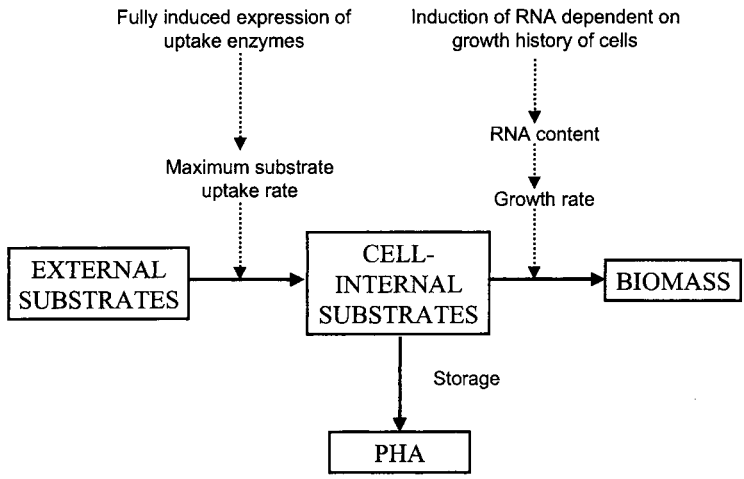

Figure 4. Schematic representation of the substrate flux in a microbial cell and the factors which influence the metabolic pathway (after Krishna *et al.* 1999 [20]).

REFERENCES

1. Henze M, Grady CPL, Gujer W, Matsuo T, Marais GvR (1995) Activated sludge model No.1, IAWPRC -STR no1 IWA London ISSN:1010-707X
2. Van Loosdrecht MCM, Pot MA, Heijnen JJ. (1997) Wat. Sci. Technol. 35:41-47.
3. Zevenhuizen LPTM, Ebbink AG. (1974) Ant. Leeuwenh. 40:103-120
4. Dircks K, Pind P. F., Mosbæk H, and Henze M. (1999). Water SA 25 (1), 69-74.
5. Dircks K, Beun JJ, Van Loosdrecht MCM, Heijnen JJ, Henze M (2000) Glycogen metabolism in aerobic mixed cultures. Submitted
6. Van Loosdrecht MCM, Smolders GJ, Kuba T., Heijnen JJ (1997) Ant. van Leeuwehoek 71:109-116
7. Cech JS, Hartman P (1993) Wat.Res. 27:1219-1225.
8. Chudoba J., Grau P, Ottova V (1973) Wat. Res. 7:1389-1406.
9. Van den Eynde E, Vriens L, Wynants M, Verachtert H. 1984.. Appl. Microbiol. Biotechnol. 19:44-52.
10. Ulrich AH, Smith (1951) Sewage and Ind. Wastes 23:1248-1255.
11. Gujer W, Henze M, Takashi M, Van Loosdrecht MCM. (1999). Wat. Sci. Technol. 39:183-193.
12. Van Aalst-van Leeuwen MA, Pot MA, Van Loosdrecht MCM, Heijnen JJ. (1997). Biotechnol. Bioeng. 55:773-782.

13. Roels JA. (1983) Energetics and kinetics in biotechnology. Amsterdam: Elsevier Biomedical Press. 330 p.
14. Smolders GJF, Van der Meij J, Van Loosdrecht MCM, Heijnen JJ. (1994). Biotechnol. Bioeng. 44:837-848.
15. Beun JJ, Paletta F, van Loosdrecht MCM, and Heijnen JJ (2000a). Biotechnol. Bioeng. 67, 379-389
16. Beun JJ, Verhoef EV, Van Loosdrecht MCM, Heijnen JJ (2000b). Biotechnol. Bioeng. 68 (5), 496-507
17. Stouthamer AH. (1973). Ant. Leeuwenh. 39:545-565.
19. Murnleitner E, Kuba T, Van Loosdrecht MCM, Heijnen JJ. (1997). Biotechnol. Bioeng. 54:434-450.
20. Krishna C, Van Loosdrecht MCM. (1999). Wat. Res. 33:2374-2382.

Synthesis of Biopolyesters by Microorganisms in Activated Sludge

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Polyhydroxyalkanoates (PHAs) are biopolyesters produced by procaryotic microorganisms. They are attracting interest of researchers as environmentally friendly material because PHAs are produced from renewable resources and are completely biodegradable plastic. In this paper, studies on PHA production by using microorganisms in activated sludge are reviewed, and its perspectives are discussed.

1. INTRODUCTION

Polyhydroxyalkanoates, PHAs, are biopolyesters which are produced by microorganisms. They are thermoplastic with complete biodegradability. Because of their completely biodegradability, and they are produced from renewable resources, they are attractive materials with environmentally friendly characteristics.

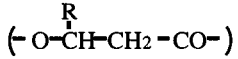
One of the most well-known PHAs is PHB, the homopolymer of 3-hydroxybutyrate (3HB). It was discovered in the middle of 1920s. In the early 1980s, ICI (Imperial Chemical Industries) started the commercial manufacturing of the copolymer of 3HB and 3-hydroxyvalerate (3HV), with the trade name of Biopol. The copolymer of 3HB and 3HV, or P(3HB-co-3HV), has elastic physical property than PHB, and had a wider applicability in the market. But at this moment, P(3HB-co-3HV) has not achieved a wide market because it is several to tens of times more expensive than other petrochemically produced plastics such as polyethylene, polypropylene, and polyvinylchloride[1, 2]. In order to reduce production cost, different effort have been made which include the use of recombinant microorganisms[3, 4] and the introduction of PHA synthetic genes to plants [5].

It is widely recognized that there are PHA accumulating microorganisms in activated sludge [6]. One of the examples is the metabolisms of polyphosphate accumulating

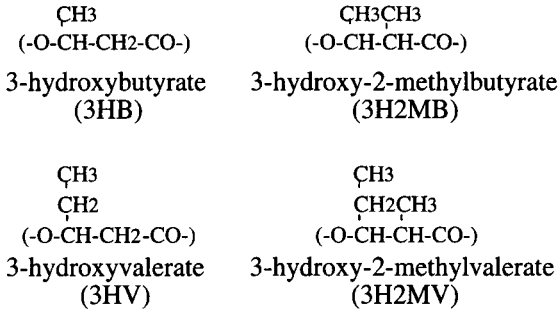
organisms (PAOs) in the enhanced biological phosphorus removal (EBPR) activated sludge processes. They accumulate PHA by taking up volatile fatty acids under anaerobic conditions. Our group has been seeking a way to utilize activated sludge for the production of PHAs. We expect that PHA production by activated sludge have the following advantages [7, 8].

(1) By using activated sludge, waste organic materials in solid wastes or wastewaters can be recovered and reused as biodegradable plastics.
(2) The cost of PHA production can be reduced; (a) the cost for the production of biomass that produce PHAs is reduced because PHAs are produced by waste sludge, (b) the cost for raw materials of PHAs is reduced if waste materials are used as the raw materials, and (c) the cost of reactor construction and operation is reduced because sterilization is not required.
(3) PHAs which is not produced by known pure cultures could be obtained by using activated sludge instead of pure cultures. The examples are PHAs containing 3-hydroxy-2-methylbutyrate (3H2MB) and 3-hydroxy-2-methylvalerate (3H2MV) as shown in Figure 1, which have been reported only in anaerobic-aerobic activated sludge, not in pure cultures [9].

In addition to the advantages above, it should be taken into account that activated sludge is a very important source of information on the PHA synthetic metabolisms in microorganisms



(A) Basic structure of PHA monomeric unit found in natural environment.



(B) PHA monomeric units found in anaerobic-aerobic activated sludge.

Figure 1. Chemical structures of PHA monomeric units.

which have not yet been observed in pure cultures. The typical example is the formation of PHAs which contain 3H2MB and 3H2MV as is described above. We expect that in activated sludge, there must be more diversed mechanisms of PHA production than which have been observed in pure cultures. For the basic research on PHA synthesis by microorganisms, activated sludge is a very interesting and attractive field, where unknown PHA metabolisms can be discovered.

Our group has been investigating on the use of activated sludge for the production of PHAs by putting focus on the increase of PHA content of activated sludge. It is because the production cost of PHAs is very much dependent on the cost of PHA recovery from biomass which is affected by the PHA content of biomass. In 1995, our group reported 62% of PHA content in activated sludge[8, 10]. But at this moment, the factors which affect PHA production capability of activated sludge is not well identified.

In this paper, studies on PHA production by activated sludge are reviewed, and discussion will be made about what should be investigated in order to realize PHA production by activated sludge.

2. STUDIES ON PHA PRODUCTION BY ACTIVATED SLUDGE

2.1 Attempts to increase PHA content of activated sludge

In order to reduce the cost of PHA production, it is essential to increase the PHA content of activated sludge. Our group has been trying to increase the PHA content of activated sludge by applying different acclimatization method and different conditions for PHA production [8]. In order to achieve higher PHA content, having a side stream reactor for PHA production rather than main stream PHA production is beneficial, as shown in Figure 2. That is, the process is divided into two reactors; the PABER, PHA accumulating bacteria enrichment reactor, and the PPR, PHA production reactor.

Although it is well known that PHA is accumulated by anaerobic-aerobic activated sludge under anaerobic conditions, PHA production under anaerobic conditions is limited by the availability of energy accumulated as polyphosphate and/or glycogen. It has been demonstrated by Iwamoto *et al.*[11] that anaerobic-aerobic activated sludge accumulate PHA not only under anaerobic conditions but also under aerobic conditions. Though introduction of air supply to PHA synthesis adversely affect the yield of PHA from substrate, more PHA is produced by the supply of energy produced by oxidative phosphorylation. Iwamoto *et al.* [11] reported that produced PHA per unit of initial MLVSS increased linearly with the increase of oxygen supply up to 3 mgO/gMLVSS/min, and higher oxygen supply did not positively affect PHA production. The rate of PHA production was found to be the maximum in the initial several hours, then it decreased quickly and PHA production was almost ceased after 24hours.

In order to further increase PHA content of activated sludge, Iwamoto *et al* .[10] tried a

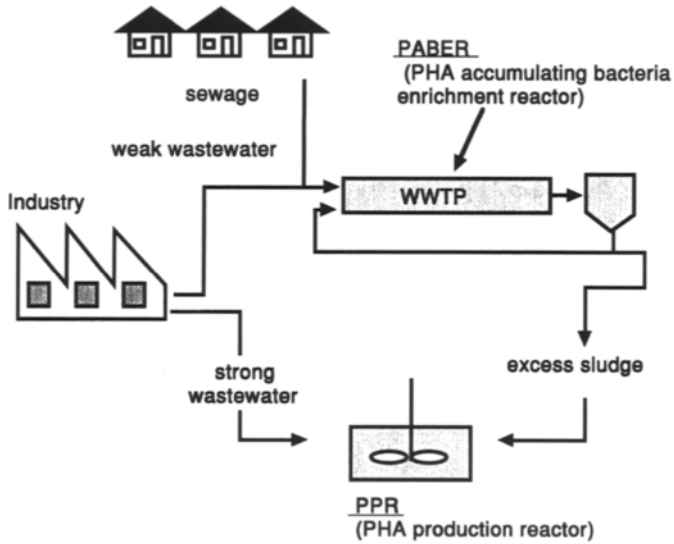


Figure 2. Side stream PHA production system composed of a PABER and a PPR.

new activated sludge process in the attempt to further enrich PHA accumulating microorganisms. They introduced as PABER the microaerophilic-aerobic process, in which a little amount of oxygen was introduced into the anaerobic stage of anaerobic-aerobic process. Their idea was to help the growth of PHA accumulating organisms which do not have the ability to accumulate polyphosphate or glycogen which are necessary for the anaerobic substrate uptake. Iwamoto *et al.*[10] reported that microaerophilic-aerobic sludge had the capability to aerobically accumulate PHA up to 62 % of MLSS . They expected that under microaerophilic condition, the substrate is taken up by PHA accumulating microorganisms while ATP and reducing power is provided by aerobic metabolisms. But what they found in the microaerophilic-aerobic process was no substrate uptake in the microaerophilic condition. They did not have rational reasoning for the effect of microaerophilic condition for the enrichment of PHA accumulating microorganisms.

Takabatake *et al.* [12] investigated the reproducibility of the effect of microaerophilic-aerobic process to increase the PHA accumulation capability of activated sludge. They operated sequencing batch reactors under the same conditions except for the method of oxygen supply, fully aerobic, anaerobic-aerobic, and microaerophilic-aerobic. They reported that PHA accumulation capability of sludge was 20 to 30 % for fully aerobic sludge, 17-57 % for anaerobic-aerobic sludge, and 33-50% for microaerobic-aerobic sludge. What they found was that anaerobic-aerobic activated sludge exhibited as high PHA accumulation capability as microaerophilic-aerobic sludge did, while it was not very stable.

The works by Iwamoto *et al.* [10] and Takabatake *et al.* [12] were conducted with

sequencing batch reactors fed with synthetic wastewater as substrate containing acetate, propionate, peptone, and yeast extract as the main carbon sources. In their studies, the effect of oxygen supply to the enrichment of PHA accumulating bacteria in PABER was focused mainly. The effect of other factors on PABER such as HRT, SRT, time sequence of operation, loadings, pH, temperature still remains unstudied. The conditions of PHA accumulation reaction in PPR has not yet been studied in detail, except for the effect of oxygen supply and the coexistence of nitrogenous compounds.

Takabatake *et al.* [13] conducted a field survey on PHA production by full scale activated sludge. Activated sludge from four full scale treatment plants with different streamlines were sampled, transported to the laboratory, and acetate was fed under aerobic condition. The PHA content was measured after 24 hours of aerobic incubation. The average PHA content after the aerobic incubation was 18.6 %, with the minimum of 6.2 % to the maximum of as high as 29.5%. They compared the PHA production capability of activated sludge from different operational conditions in the same wastewater treatment plant; they found that activated sludge in conventional process had a higher PHA accumulation capability than that in anaerobic-aerobic process.

Having all the outcomes above described, it is rather difficult to determine the strategy to increase PHA accumulation capability of activated sludge, while many factors still remains unstudied.

2.2 Stability of PHA content achieved in PPR

For the commercial production of PHA, not only the maximum but also the stability of PHA content achieved in PPR is important. According to our experience, microaerophilic-aerobic process tends to be unstable in operation because of bulking by the predomination of filamentous bacteria [10]. Even if microaerophilic-aerobic process is operated steadily, it seems it is not easy to achieve high and stable PHA production in PPR. The operation of PABER reported by Takabatake *et al.* [12] shows rather unstable PHA accumulation capability of activated sludge. The stability of PHA accumulation capability needs to be studied more.

2.3 Production of PHAs with unique characteristics

Research on PHA production by activated sludge needs not be limited to the direction of increasing PHA content. Interesting PHAs are known to be produced especially by anaerobic-aerobic activated sludge under anaerobic conditions.

As was described in introduction, PHAs with 3H2MB and 3H2MV units have never been found in pure cultures. There are two unique points about PHAs with these two units. The first thing is that these two units have two branches from the main chain, while other PHAs produced by pure cultures have only one branch at the maximum. Another interesting point is that 3H2MB and 3H2MV unit have two chiral centers from the stereochemical point of view. The additional branch is supposed to have an effect to protect ester bond from lysis. At this

moment, the physical properties of PHA which contain 3H2MB or 3H2MV have not yet been done. Detailed investigation on their physical property should be done. Also, it must be interesting to see its degradation profile under different conditions, because the control of degradation of biodegradable polymers is important when we think about the practical situation of its application.

It is expected, based on the PHA synthetic pathways of pure cultures so far clarified, that PHA monomeric units such as 3H2MB and 3H2MV are produced by the condensation of acetyl-CoA and propionyl-CoA by an enzyme called ketothiolase, then reduced and polymerized to form PHA as shown in Figure 3. The condensation step can be explained by Claisen condensation [14]. The reason why 3H2MB or 3H2MV are not produced by pure cultures can be explained by the difference of ketothiolase; we are expecting that ketothiolase in pure cultures so far studied cannot accept propionyl-CoA as substrate B. Ketothiolase in anaerobic-aerobic activated sludge is interesting because it can use propionyl-CoA in addition to or instead of acetyl-CoA.

The accumulation of PHA containing 3H2MB and 3H2MV is not always observed. In our experience, activated sludge which was acclimatized with synthetic wastewater without propionate did not have the ability to accumulate PHA containing 3H2MV. Instead, the sludge was able to accumulate 3HV rich PHA (about 80 % in molar basis) from the mixture of acetate and propionate [15].

Thinking about the mechanism of the reaction, we may well expect the existence of ketothiolase which can use butyryl-CoA for substrate B in Figure 3, and we might well expect the formation of PHA containing 3-hydroxy-2-ethylalkanoate. But the formation of this kind of monomeric units in PHA has not yet been reported.

The fate and the biochemical pathways of volatile fatty acids other than acetate and propionate by anaerobic-aerobic activated sludge under anaerobic conditions is still to be studied more. In our experience, anaerobic uptake of mixture of acetate and butyrate resulted in PHA rich in 3-hydroxycaproate, though the reproducibility is not yet examined.

When we turn our attention to aerobic production of PHA by activated sludge, Iwamoto *et al.* reported that volatile fatty acids taken up by microaerophilic-aerobic activated sludge under aerobic condition was found to be accumulated only as 3HB and 3HV[10]. It might be rather difficult to expect the formation of unique PHAs if PHA is to be produced under aerobic condition.

2.4 Physical properties of PHAs produced by activated sludge

The chemical and physical properties of PHAs and its stability is another matter of concern about PHAs produced by activated sludge. It can be expected that PHAs produced by mixed culture is more diversed than that produced by pure culture. The PHA product from activated sludge might be less reliable and less stable in quality when compared with PHA from pure cultures. But limited number of research has been done on the physical and chemical properties of PHA from activated sludge. Inoue *et al.* [16] reported that PHA produced by

2.5 Factors which Limits maximum PHA content

While continuing work to increase PHA accumulation capability of activated sludge, it is needed to clarify why there is a limit of PHA content in microorganisms. In the case of activated sludge, we also need to clarify how many percentages of microorganisms in activated sludge are contributing to PHA accumulation. We will be able to increase PHA content of activated sludge by increasing either or both of (1) PHA content of individual cells and (2) percentage of PHA accumulating organisms in activated sludge. In the studies on PHA production by activated sludge so far performed, these two factors have never been separately discussed clearly.

It is not very well clarified why there is a limit in PHA content in microorganisms. In order to increase PHA content of individual cells, there have been reports that addition of nutrients such as yeast extract is effective.

In the case of activated sludge, not only the initial percentage of PHA accumulating organisms in activated sludge but also the growth of non-PHA accumulating organisms in the course of incubation for PHA production is another matter of concern. Especially, the existence of nutrients in raw materials may cause the growth of microorganisms rather than PHA production. Ueno *et al.* [19] and Takabatake *et al.* [12] reported that PHA production by activated sludge acclimatized in anaerobic-aerobic process was adversely affected by the coexistence of ammonia but the reduction of PHA production was less than 30 %.

2.6 Yield of PHA from raw materials

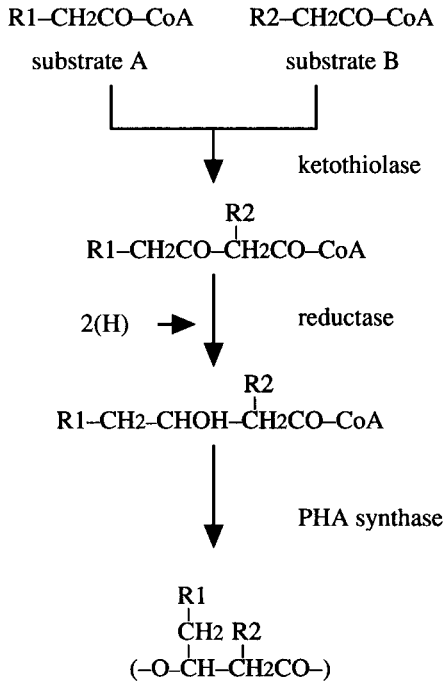
Theoretical yield of acetate into PHB is expected to be 95.6% (w/w basis) under anaerobic conditions if the reducing power is supplied by the glycolysis of glycogen, as reaction (1). The yield of PHA obtained from acetate under anaerobic condition was almost similar to the theoretical [9, 20].

$$6\ CH_3COOH + (C_6H_{10}O_5)_n \rightarrow 4\ 3HB + (C_6H_{10}O_5)_{n-1} \quad (1)$$

Theoretical yield of PHB from acetate under aerobic condition can be estimated as follow. Under aerobic condition, some part of acetate is oxidized by TCA cycle to yield ATP and reducing power equivalent to NADH or NADPH. The degradation of acetate by TCA cycle is described as follow.

$$1\ \text{acetate} + 1\ ATP \rightarrow 1\ \text{acetyl-CoA} \rightarrow 2\ CO_2 + 3\ NADH + 1\ FADH + 1\ ATP \quad (2)$$

Since FADH is a weaker reductant than NAD(P)H, here we assume that FADH is not used for the reduction of acetoacetyl-CoA into 3-hydroxybutyryl-CoA but is used for oxidative phosphorylation. In oxidative phosphorylation from one molecule of FADH yields 2 molecules of ATP. Then, reaction (2) is described as follow.



R1 or R2 = H : acetyl-CoA
R1 or R2 = CH_3 : propionyl-CoA

R1	R2	end product
H	H	3HB
CH_3	H	3HV
H	CH_3	3H2MB
CH_3	CH_3	3H2MB

Figure 3. Formation of PHA monomeric units.

anaerobic-aerobic activated sludge fed with propionate under anaerobic condition had a number average molecular weight of $\overline{M}_n = 1.6 \times 10^5$ to 1.8×10^5 with polydispersity index $\overline{M}_w / \overline{M}_n = 2.5$ to 2.9. They also reported that the PHA could be fractionated by using water/acetone mixed solvent into fractions by differences in average molecular weights as well as in monomer compositions. But Yoshie *et al.* [17] suggests that even PHA produced by pure cultures can be fractionated into fractions with different average molecular weighs or different monomer compositions.

Lemos *et al.*[18] reported the $\overline{M}_w$, weight average molecular weight of PHAs produced by activated sludge from acetate, propionate, and butyrate to be 2.5×10^5 to 1×10^6 under anaerobic conditions, with the polydispersity of around 2.

As far as we know, these are only reports on the physical properties of PHAs produced by activated sludge. In relation with the stability of PHA accumulation capability, the stability and the control of physical properties of PHAs need to be investigated.

1 acetate + ATP --> 2 CO_2 + 3 NADH + 3 ATP (3)

If remaining 3NADH are to be oxidized, nine more ATP will be produced, because each molecule of NADH will yield 3 molecules of ATP in oxidative phosphorylation.

1 acetate + ATP --> 2 CO_2 + 12 ATP (4)

By combining reactions (3) and (4) with the following equation on the conversion of acetate into PHA, we obtain reaction (6).

2 acetate + 2 ATP + 1 NAD(P)H --> 1 3HB (5)
81 acetate --> 33 3HB + 30 CO_2 (6)

Reaction (6) gives the theoretical yield for PHB production under aerobic condition. The yield here is 0.58 (w/w). The yield of PHA Iwamoto *et al.* [10] found in the aerobic production of PHA by anaerobic-aerobic activated sludge was around 0.27, which was significantly lower than the theoretical. This means that significant part of ATP produced and acetate taken up was used for other metabolic activities than PHA production. The use of antibiotics to suppress cell growth or the application of lower temperature to reduce loss of energy might positively affect the yield of PHA.

2.7 PHA production from glucose under anaerobic conditions

One of the most interesting metabolisms about PHA production by activated sludge is the conversion of glucose into PHA. Theoretically, the conversion of glucose into 3HV is ATP yielding reaction while redox balance is maintained, as shown in Figure 4. The reaction may well be called 3HV fermentation [9, 20, 21]. The metabolisms of so called G bacteria can be explained by 3HV fermentation from glucose. And the metabolisms of GAOs, glycogen accumulating organisms, is similar to 3HV fermentation with the difference of initial material being glycogen instead of glucose. The merit of using 3HV fermentation for PHA production are (1) energy for air/oxygen supply is not needed and (2) glucose is much cheaper than acetate and/or propionate. Though glucose is not usually found in wastewater, the use of 3HV fermentation is still attractive if we think about the possibility of using mixed culture for PHA production from pure raw materials. While some *Rhodobacter* species are known to do a very similar metabolism and produce 3HV from glucose, the reaction goes basically under aerobic condition.

2.8 Activated sludge as a field to study basics about PHA metabolisms

Saito *et al.*[22, 23] isolated from activated sludge a strain of Comamonas acidovorans which have the ability to produce very unique PHA with very high percentage of 4-hydroxybutyrate. As the source of microorganisms with very unique PHA production

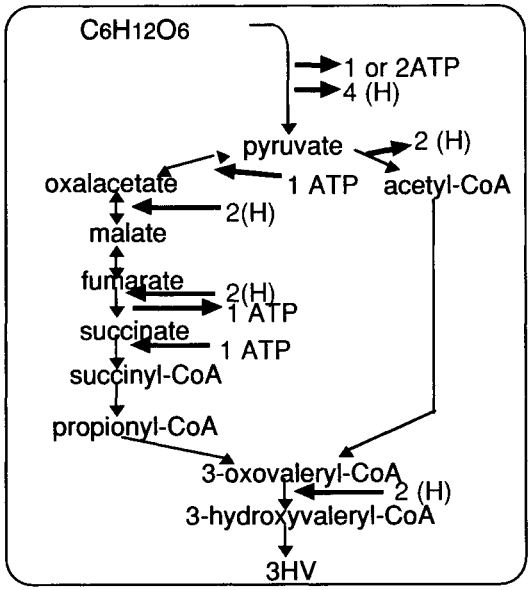


Figure 4. Proposed metabolic mechanism for 3HV fermentation.

capability, activated sludge is very attractive. But it is said that most of microorganisms in activated sludge cannot be isolated easily. While investigation on PHA in activated sludge provided us with many interesting findings about PHA metabolisms which could not have been discovered in pure culture studies, the new findings have not affected PHA related studies very much. We think that the reason for this can be attributed to the difficulty in the isolation of interesting PHA accumulating microorganisms in activated sludge. For example, even anaerobic production of PHB from acetate by polyphosphate accumulating microorganisms have never been reproduced in pure culture level.

But as the molecular tools are applied to activated sludge processes, we expect that even the identification of unculturable microorganisms will become possible and their metabolisms will be clarified. Or at least, the genes of PHA accumulating microorganisms will be clarified. Once the genes are clarified, it will be possible to introduce the gene into pure microorganism and clarify the functions of the protein coded on the gene. Here, it will also become possible to see how the functions of the enzyme is controlled. While there are certainly many many difficulties, molecular level studies on PHA accumulating microorganisms in activated sludge will enable us to identify 3H2MV producing microorganisms and further it will become possible to produce 3H2MV containing PHAs by genetically modified organisms. Also, the conversion of glucose into biodegradable plastic may become a very common method of the production of plastics.

3. CONCLUDING REMARKS

In the present society, organic compounds in wastewater is regarded as pollutant and is removed from water in such forms as carbon dioxide or waste sludge. If it is possible or if it becomes feasible to utilize activated sludge as the source of biodegradable plastic PHA, it will be a new way of material recycle in our society. That is, organic materials in wastewater will now be regarded as resources for biodegradable plastic. This idea is just a dream, at this moment, but if technology is more improved, it may be realized in the future.

As a more achievable goal, it is interesting to put goal at utilizing mixed culture for PHA production. In this case, production of PHAs which cannot be produced by pure cultures is a very interesting topic to pursue. In addition, clarifying PHA accumulators by using different molecular tools is also interesting. Studies from different approach are awaited.

REFERENCES

1. Doi Y (1990) Microbial Polyesters. VCH Publishers, New York.
2. Anderson, A. J., and Dawes, E. A. (1990). Microbiological Reviews, 54(4), 450-472.
3. Lee, S.Y., Choi, J.i., Wong, H.H., Biological Macromolecules, 25(1-3), 31-36, 1999.
4. Kichise, T., Fukui, T., Yoshida, Y., and Doi, Y. Biological Macromolecules, 25(1-3), 69-77, 1999.
5. Valentin, H.E., Broyles, D.L., Casagrande, L.A., Colburn, S.M., Creely, W.L., Delaquild, P.A., Felton, H.M., Gonzalez, K.A., Houmiel, K.L., Lutke, K., Mahadeo, D.A., Mitsky, T.A., Padgette, S.R., Reiser, S.E., Slater, S., Stark, D.M, Stock, R.T., Stone, D.A., Taylor, N.B., Thorne, G.M., Tran, M., and Gruys, K.J. Biological Macromolecules, 25(1-3), 303-306, 1999.
6. Mino, T., van Loosdrecht, M.C.M., and Heinen, J.J., Water Research, 32(11), 3193-3207, 1998.
7. Satoh, H., Iwamoto, Y., Mino, T., and Matsuo, T. Water Science and Technology, 38(2), 103-109, 1996.
8. Satoh, H., Mino, T., and Matsuo, T. Biol. Macromol., 25(1-3), 105-109, 1999.
9. Satoh, H., Mino, T., and Matsuo, T. Wat. Sci. Tech., 26(5/6), 933-942, 1992.
10. Iwamoto, Y., Satoh, H., Mino, T., and Matsuo, T.Journal of Environmental Systems and Engineering, No. 573/VII-4, 19-26, 1997. (in Japanese)
11. Iwamoto, Y., Satoh, H., Mino, T., and Matsuo, T. Proc. of Environmental Engineering Research, Vol. 32, pp329-338, 1994. (in Japanese)
12. Takabatake, H., Satoh, H., Mino, T., and Matsuo, Conference Preprint Vol. 2, Asian Water Qual '99, 7th IAWQ Asia-Pacific Regional Conf., Taipei Taiwan, 1175-1180, 1999.
13. Takabatake, H., Satoh, H., Mino, T., and Matsuo, Environmental Engineering

Research, Vol. 36, 137-144. (in Japanese)
14. Morrison, R.T., and Boyd, R.N., Organic Chemistry, 4th Ed., Allyn and Bacon, Inc., Newton, Massachusetts, USA, 1983.
15. Satoh, H., Mino, T., and Matsuo, T., Proc. of Environ. & Sani. Eng. Research, Vol.28, pp39-49, 1992. (in Japanese)
16. Inoue, Y., Sano, F., Nakamura, K., Yoshie, N., Satoh, H., Mino, T., Matsuo, T., and Doi, Y., Polymer International, 39, 183-189, 1996.
17. Yoshie, N and Inoue, Y. Biological Macromolecules, 25(1-3), 193-200, 1999.
18. Lemos, P.C., Viana, C., Salgueiro, E.N., Ramos, A.M., Crespo, J.P.S.G., and Reis, M.A.M., Enzyme and Microbial Technology, 22, 662-671, 1998.
19. Ueno, T., Satoh, H., Mino, T., and Matsuo, T. Polymer Preprints, Japan 42, 3673-3675. (in Japanese)
20. Satoh, H., Mino, T., and Matsuo, T., Wat. Sci. Tech., Vol.30, No.6, 203-211, 1994.
21. Liu W.T, Mino, T., Nakamura, K., and Matsuo, T. J. Ferment. and Bioeng., 1994;77:535.
22. Saito, Y. and Doi, Y. Int. J. Biol. Macromol. 16(2), 99-104, 1994.
23. Saito, Y., Nakamura, S., Hiramitsu, M., and Doi, Y. Polym. Int. 36(3), 169-174, 1996.

Material recovery from wastewater using photosynthetic bacteria

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A material recovery process from wastewater using photosynthetic bacteria was introduced in this paper. Photoheterotrophic growth of photosynthetic purple nonsulfur bacteria results in high conversion rates of dissolved organic matter into their cell materials, such as protein and PHA (polyhydroxyalkanoate), which can be utilized for agricultural and industrial uses.

Growth rates and PHA accumulation performances of *Rhodobacter sphaeroides* were determined under various conditions (substrate, temperature, light intensity and sulfide concentration). Optimal light intensity and temperature were shown to be >6000 lx and 25-35°C, respectively. Acetate was preferable for the growth and PHB production, while propionate gave no accumulation of PHB (Poly-3-hydroxybutyrate). The growth of *R. sphaeroides* was sensitive to sulfide, but this problem of growth inhibition could be alleviated by adding purple *sulfur* bacteria *Chromatium vinosum*.

1. INTRODUCTION

Photoheterotrophic growth of purple nonsulfur bacteria, a kind of photosynthetic bacteria, brings about high conversion rates of dissolved organic matter into their cell materials and little emission of carbon dioxide. From this point of view, wastewater treatment using purple nonsulfur bacteria under light and anaerobic conditions is expected to produce a large amount of useful biomass with little carbon dioxide, one of the major green-house gases (Figure 1). The biomass of these bacteria can be utilized for agricultural and industrial uses such as a feed for fish and animals, fertilizers [1] and source of PHAs (polyhydroxyalkanoates) [2]. Polyhydroxyalkanoates are well known to be stored intracellularly by various bacteria under imbalanced nutrient conditions and is commercially produced into a biodegradable plastic.

The system of wastewater treatment and biomass production is shown in Figure 2.

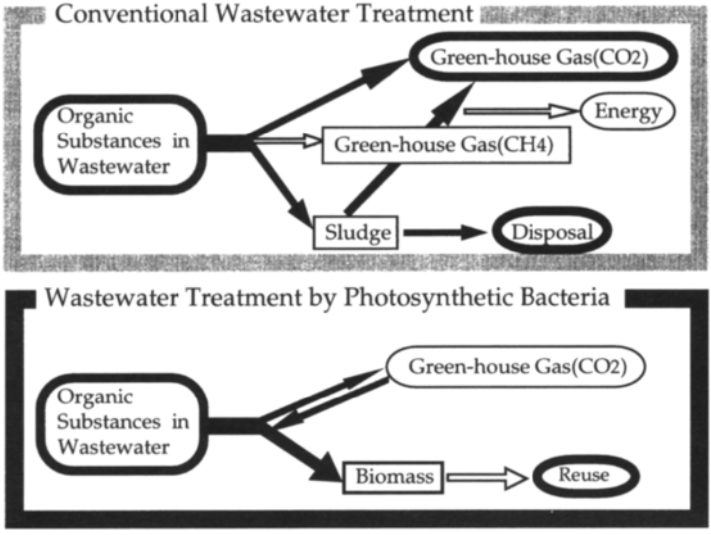

Figure 1. Scheme of carbon conversion.

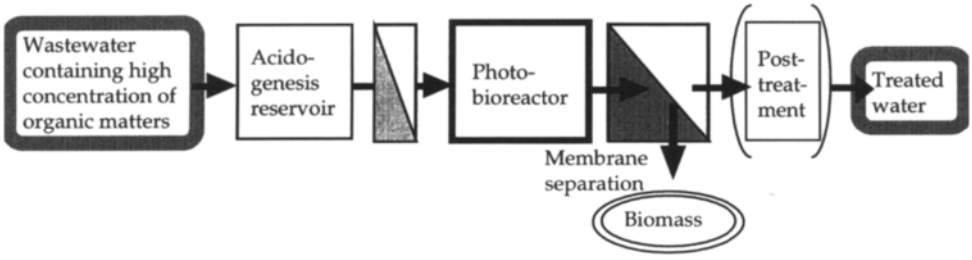

Figure 2. The system of wastewater treatment and biomass production.

The following points should be investigated :

*how to achieve the suitable operational conditions,

*how to control the bacterial population in the system,

*how to irradiate photosynthetic bacteria, or the design of a photobioreactor, and

*how to harvest the biomass.

This work is concerned with the first two points.

For the former, the growth characteristics of purple nonsulfur bacteria should be discussed. Many researchers have been investigating PHA production by purple nonsulfur bacteria under nitrogen-limited conditions [2,3,4,5]. Under such conditions, PHA would be accumulated and would never be converted into other cell materials. However, since some wastewater contains sufficient nitrogen compounds, we must investigate PHA accumulation/consumption under nitrogen-*un*limited conditions. In this study, batch experiments were carried out using purple nonsulfur bacterium *Rhodobacter sphaeroides* to determine its growth rates and PHA

accumulation performances under various conditions (substrate, temperature, light intensity and sulfide concentration).

Pure culture must be easily controlled for the production of PHAs, but in practice, pure cultivation in wastewater is difficult. Therefore, the effect of the presence of other bacteria and the possibility of a mixed culture system should be investigated. As presented in this paper, purple nonsulfur bacteria, the main contributors in this system, are sensitive to sulfide, which is likely to be produced by sulfate-reducing bacteria under anaerobic conditions. We tested a mixed culture system with purple *sulfur* bacteria to alleviate growth inhibition by sulfide.

2. MATERIALS AND METHODS

2.1. Inoculum and medium

The inoculum of purple nonsulfur bacterium was *Rhodobacter sphaeroides* IFO 12203. The culture medium for all experiments was prepared according to the method of Hoshino and Sato [6]. Ammonium concentration was 212 mgN/L in this medium. Assuming the cell composition of $C_5H_7O_2N$, this ammonium concentration would be sufficient for up to 908 mgC/L of carbon assimilation.

To prepare the mixed culture system, purple sulfur bacterium *Chromatium vinosum* ATCC 17899 and sulfate-reducing bacterium *Desulfovibrio baarsii* ATCC 33931 were inoculated.

2.2. Growth factors

Specific growth rates of *R. sphaeroides* IFO 12203 under various conditions were determined. Light intensity, temperature, substrate and sulfide concentration were examined as growth factors. As shown in Figure 3, batch experiments were conducted using screw-capped glass tubes (approximately 5 mL volume), which were originally designed for fluorescent spectrophotometry, as culture vessels that enables us to monitor the absorbance without sampling. After inoculation, they were

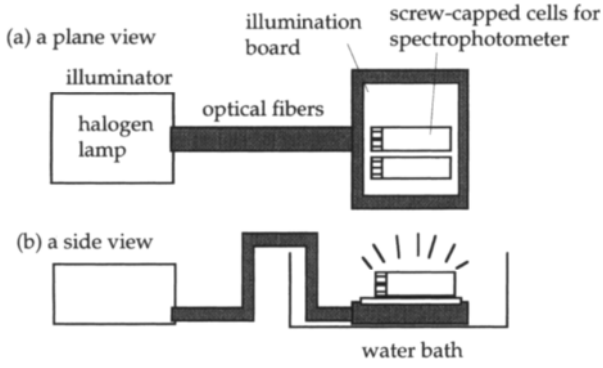

Figure 3. Batch experiment to evaluate growth factors.

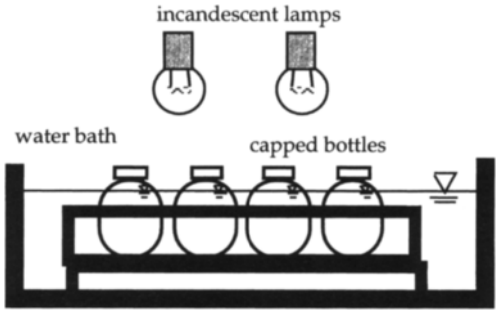

Figure 4. Batch experiment to investigate PHA accumulation performance and to test mixed culture system.

put on an illumination board which was connected to a halogen lamp with optical fibers, in a water bath. Specific growth rate was calculated from the increase of absorbance at 770 nm in the logarithmic growth phase.

2.3. PHB accumulation

Poly-3-hydroxybutyrate (PHB) accumulation by *R. sphaeroides* IFO 12203 was investigated. Substrates used in this experiment were sodium acetate (3 hydrate), sodium propionate, sodium n-butyrate and glucose. Sodium hydrogen carbonate was added in the cases of using propionate and n-butyrate because *R. sphaeroides* needed it to assimilate the organic matters [7].

Five or more bottles (approximately 70 mL volume) were prepared for each experiment. After autoclaving, 1 mL of precultivated *R. sphaeroides* was inoculated. Following one-day storage under a dark condition to consume trace amounts of dissolved oxygen, the bottles were put in a water bath under incandescent lamps (Figure 4). Cell, protein and sugar concentrations in terms of carbon concentration [mgC/L] were determined at each stage of the growth. Cells were harvested by centrifugation and were lyophilized to determine intracellular PHB content. Poly-3-hydroxybutyrate concentration was also expressed by the unit of mgC/L.

2.4. Mixed culture system

To alleviate growth inhibition by sulfide, purple sulfur bacterium *C. vinosum* was introduced. An experiment to test this mixed culture system was conducted by adding sodium sulfide to the culture medium. Although we also inoculated sulfate-reducing bacteria, we could neglect their growth in this experiment. Cell concentration for each bacterium was evaluated based on microscopic observation [8].

2.5. Assay

Total organic carbon (TOC) was analyzed using a TOC analyzer (TOC-500, Shimadzu, Japan). Acetate, propionate and n-butyrate concentrations were analyzed by HPLC/UV. Poly-3-hydroxybutyrate was quantified by GC/FID after

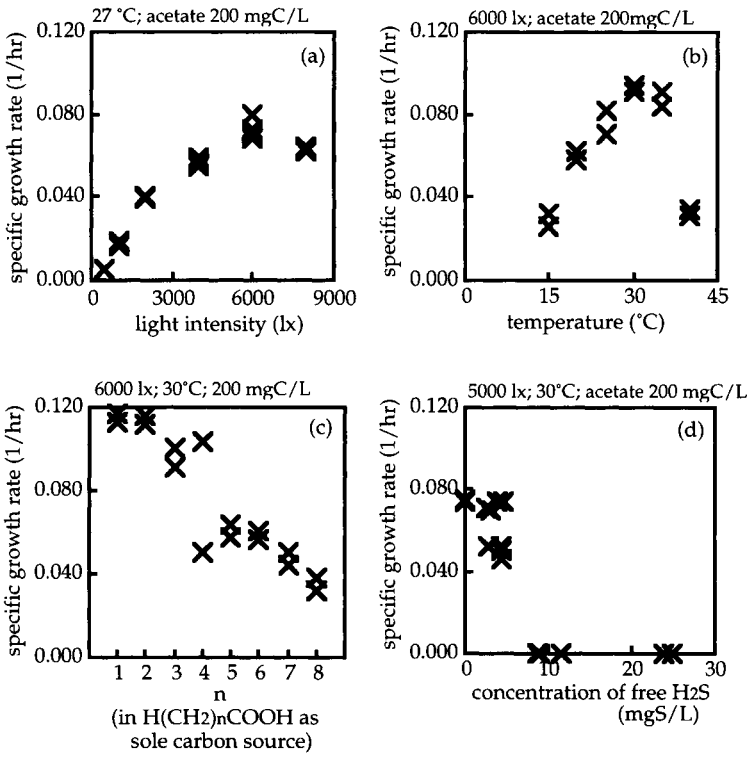

Figure 5. Growth factors of *Rhodobacter sphaeroides*.
(a)light intensity, (b)temperature, (c)carbon source, (d)sulfide

methanolysis [9]. The amount of protein was quantified by the Lowry method and that of sugar was quantified by the anthron method. Sulfide concentration was quantified by the methylene blue method.

3. RESULTS AND DISCUSSION

3.1. Growth factors

Figure 5 shows specific growth rates of *R. sphaeroides* under various conditions of light intensity, temperature, substrate and sulfide concentration.

Figure 5(a) indicates light saturation at around 6000 lx. Figure 5(b) shows that the temperature must be kept at 25-35°C. These parameters should be considered when designing the photobioreactor.

As shown in Figure 2, acidogenesis of wastewater is expected in the material recovery system of photosynthetic bacteria. The composition of organic acids, which is related to the performance of acidogenesis, influenced the growth of *R. sphaeroides*, as shown in Figure 5(c). This result indicates that acetate (n=1), propionate (n=2) and

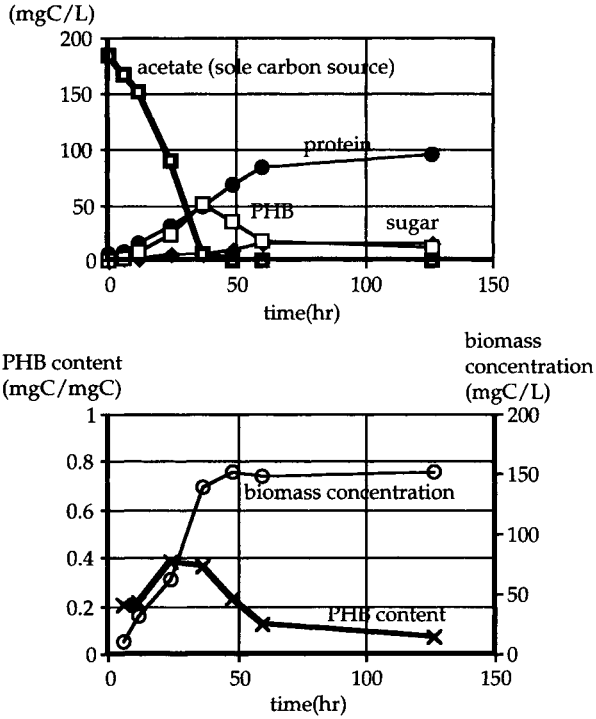

Figure 6. Growth of *Rhodobacter sphaeroides* and variation of their cell composition and PHB content.

Table 1.
PHB content of *Rhodobacter sphaeroides* grown on various carbon source

carbon source	feed concentration (mgC/L)	maximum PHB content (mgC/mgC)
acetate	200	39
acetate	400	52
propionate	200	0
n-butyrate	200	9
acetate + propionate + n-butyrate	100 + 100 + 100	21
glucose	200	2

components (PHB, protein and sugar) and PHB content are shown in this figure. Poly-3-hydroxybutyrate was consumed after substrate depletion since the bacteria were fed a sufficient amount of nitrogen to convert PHB into other cell materials such as protein. In this case (acetate as the sole carbon source), the PHB content reached up to 39% in the phase of acetate assimilation.

Poly-3-hydroxybutyrate was accumulated with growth in these experiments, although Brandl *et al.* had shown that PHB accumulation occurred mainly in the stationary phase under an ammonium-limited condition [3]. Their results probably indicate that PHB accumulation occurred with the degradation of the other components in the stationary phase because the total mass of cells did not change. Such degradation of the other components might be caused by high pH (final pH in their experiments was 9.8). In our experiments, PHB accumulation occurred with an increase of the total cell mass because the amount of pH buffer was sufficient.

Poly-3-hydroxybutyrate contents of *R. sphaeroides* grown with various carbon sources are summarized in Table 1. Poly-3-hydroxybutyrate content strongly depends on the type and the amount of carbon source. Acetate gave a high PHB content while propionate a low PHB content. According to these results, pretreatment in which organic matter in wastewater is converted into acetate is advantageous for PHB production. The presence of other substrates (propionate, n-butyrate and glucose) will decrease the PHB content and, at the same time, increasing the protein content.

In order to obtain cells with higher PHB content, treatment of wastewater should not be completed because substrate depletion causes PHB degradation into other cell components such as protein. This method requires a post-treatment in order to treat wastewater completely; the protein-producing process is a possible post-treatment. When the original wastewater contains little nitrogen and pretreatment of the organic matter in the wastewater into acetate can be achieved, PHB production should be easily realized. In such a case, PHB accumulators would not degrade PHB by themselves and the PHB content can be kept high.

3.3. Mixed culture system

As shown in Figure 5(d), purple nonsulfur bacterium *R. sphaeroides* is sensitive to sulfide. If we could select raw wastewater containing little sulfur compounds, this high sensitivity could be disregarded. However, sulfur compounds exist ubiquitously as sulfide itself, sulfate or in organic matters such as protein. They might inhibit the growth of purple nonsulfur bacteria. Here, we must discuss countermeasures against such inhibition, for example, pH control, or sulfide oxidation by O_2 or other bacteria. Control of the pH is one of the easiest ways of moderating the inhibition because sulfide has an ion equilibrium depending on the pH, and the free H_2S concentration decreases with increasing pH. The free H_2S concentration at pH 8 can be calculated to be about 20% of that at pH 7. Sulfide can be easily oxidized by O_2. In our previous experiment [10], even slight aeration caused a decrease in the PHB content of *R. sphaeroides*. In the case of PHB production from wastewater, precise O_2 control would be needed. The use of mixed culture of purple nonsulfur bacteria and sulfur bacteria is interesting. Photosynthetic sulfur bacteria anaerobically oxidize sulfur compounds into elemental sulfur and sulfate. This

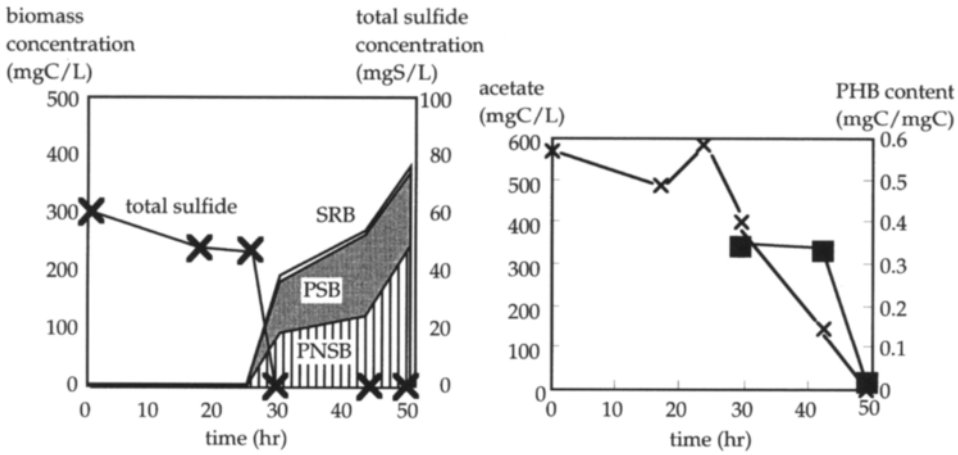

Figure 7. Mixed culture system of purple nonsulfur bacteria (*Rhodobacter sphaeroides*) and purple sulfur bacteria (*Chromatium vinosum*).
PNSB : purple nonsulfur bacteria, PSB : purple sulfur bacteria, SRB : sulfate reducing bacteria(not grown in this case)

group of bacteria can exist in a similar environment as purple nonsulfur bacteria--anaerobic and in light.

Based on this background, a mixed culture of purple nonsulfur bacteria and sulfur bacteria was tested. The result is shown in Figure 7. *Rhodobacter sphaeroides* and *C. vinosum* grew with sulfide depletion after 29 hours. Even when the initial total sulfide concentration was 63 mgS/L, the growth of *R. sphaeroides* was observed. Since purple sulfur bacteria reduces the sulfide concentration, the existence of purple sulfur bacteria helps the growth of purple nonsulfur bacteria. This result shows the symbiotic bacterial system to be effective in the alleviation of sulfide inhibition.

The time courses of acetate (the sole carbon source in this batch experiment) and PHB contents are also shown in Figure 7. Acetate was exhausted, due to the growth of bacteria, at the end of this experiment. The maximum PHB content was 35%, which is lower than that in pure culture of *R. sphaeroides* (52% in Table 1). This was because of the existence of other bacteria in this system. We infer that not only *R. sphaeroides* but also *C. vinosum* grew heterotrophically, based on the calculation of the carbon balance. It has been reported that *C. vinosum* is also able to accumulate PHA up to 50% with heterotrophic growth [4], therefore PHA accumulation in higher proportions would be realized, even in the mixed culture system, with some operational modification.

4. CONCLUSIONS

A material recovery process from wastewater using photosynthetic bacteria was

introduced in this paper.

Growth rates and PHA accumulation performances of *Rhodobacter sphaeroides* were determined under various conditions (substrate, temperature, light intensity and sulfide concentration). Optimal light intensity and temperature were shown to be >6000 lx and 25-35°C, respectively. Acetate was preferable for the growth and PHB production, while propionate gave no accumulation of PHB. The growth of *R. sphaeroides* was sensitive to sulfide, but this problem of growth inhibition could be alleviated by adding purple *sulfur* bacteria *Chromatium vinosum.*

REFERENCES

1. M. Kobayashi and Y. T. Tchan, Wat. Res., 7 (1973) 1219.
2. H. Brandl, E.J. Knee Jr, R.C. Fuller, R.A.Gross and R.W. Lenz, Int. J. Biol. Macromol., 11(2) (1989) 49.
3. H. Brandl, R.A. Gross, R.W. Lenz and R.C.Fuller, Arch. Microbiol., 155 (1991) 337.
4. M. Liebergesell, E. Hustede, A. Timm, A. Steinbuchel, R.C. Fuller, R.W. Lenz and H.G. Schlegel, Arch. Microbiol., 155 (1991) 415.
5. K. Hashimoto, H. Tsuboi, S. Iwasaki and Y. Shirai, J. of Chem. Eng. of Japan, 26(1) (1993) 56.
6. Y. Hoshino and T. Sato, IV-I.2.3. Photosynthetic bacteria : in K. Yamasato *et al.* (eds), Method for Separation of Microorganisms, R&D Planning, Tokyo, 1986. (in Japanese)
7. F. Nakajima, N. Kamiko and K. Yamamoto, Wat. Sci. Tech., 35(8) (1997) 285.
8. K. Izu, F. Nakajima and K. Yamamoto, Environ. Eng. Res., 35 (1998) 439. (in Japanese)
9. JSCE Sanitary Engineering Committee (eds), Method of Environmental Bioengineering, Gihoudo Syuppan, Tokyo, 1993. (in Japanese)
10. F. Nakajima, K. Izu and K. Yamamoto, Proc. 31st Annu. Conf. JSWE (1997) 229. (in Japanese)

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§5 Water and Wastewater Treatment in Asian Tropical and Subtropical Regions

So far most of the water and wastewater treatment processes have been developed in countries having temperate climate. Our knowledge and expertise on application of these treatment processes to countries having tropical to sub-tropical climate is so far very limited. As the climate and the life style of people differ, the quality of raw water and wastewater varies very much between the regions on the earth. Therefore it is critically important to understand the characteristics of water in the specific region to find the best water treatment process and its optimum operation procedure. For this aim this chapter comprises five sections written by authors from Asian tropical and sub-tropical nations.

Fujioka *et al.* discuss the ecology of fecal bacteria in tropical soil environment. He shows that fecal bacteria can survive and grow in natural soil environment in Oahu Island, Hawaii, which suggests doubts on fecal bacteria as an indicator of fecal pollution. Fujioka proposes two hypotheses on the growth mechanism of fecal bacterial in natural soil: i.e., only a few species that acquired adaptability to the soil environment are growing, or most of the fecal bacteria can acquire this adaptability. By analyzing the metabolic reaction pattern of 48 *E. coli* isolates and 30 enterococci isolates from soil samples taken in Oahu Island, he found a variety of fecal bacterial species in the soil samples in Oahu, concluding that most of fecal bacteria are robust enough to overcome natural constraints in the Hawaii soil.

Fang *et al.* discuss the history and present uses of anaerobic digestion. Anaerobic digestion is attractive in tropical countries because of easy and cost-saving operation, energy recovery, and high temperature. Hence more than 40 % of 1200 full-scale anaerobic wastewater treatment plants are located in tropical/ subtropical countries, and the number is still growing rapidly. He explains the wastewater treatment by anaerobic digestion in selected countries: namely China, India, Philippines, Taiwan, Thailand, Brazil, and Mexico.

Ouyang *et al.* elucidate the importance of nutrient removal in tropical countries. A biological nutrient removal process with anaerobic-aerobic-anoxic-aerobic (AOAO) sequence was quite efficient in phosphorus removal, but not in nitrogen removal. A step-feed modification of AOAO process lead to a significant improvement of nitrogen removal efficiency.

Upgrading of a waste stabilization pond to a baffled reactor was discussed by Ujiang *et al.* Waste stabilization ponds (WSPs) are widely used in tropical countries. However, most of WSPs do no work as expected due to poor design and maintenance. Ujiang shows how a waste stabilization pond can be upgraded to better performance by modifying as a baffled reactor.

Constructed wetland is another alternative waste treatment process for tropical countries. On the other hand, treatment and disposal of septage generated at many on-site wastewater treatment units is an emerging concern in tropical countries. Koottatep *et al.* applied a vertical-flow constructed wetland to a septage treatment in Thailand. At a solid loading rate of 250 kg/m^2/y the removal rates of TS, COD and TKN were more than 80%. A high concentration of nitrate in the percolate was reduced significantly by ponding the constructed wetland a few days prior to discharge.

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Microbial ecology controls the establishment of fecal bacteria in tropical soil environment

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Fecal indicator bacteria such as *E. coli* and enterococci are consistently present in Hawaii's streams in concentrations exceeding recreational water quality standards. The source of these fecal indicator bacteria has been determined to be the soil where these bacteria are able to multiply and have become part of the soil microflora. The mechanisms by which these fecal bacteria are able to multiply under ambient soil conditions have not been determined. The objective of this study was to test two hypotheses by which these fecal bacteria can establish populations in the soil environment. The first hypothesis states that the soil environment is restrictive for the growth of fecal bacteria but a sub-population of fecal bacteria with metabolic characteristics adaptable to growth in soil will be selected to establish a population. The second hypothesis states that the soil environment is permissive and most strains of fecal bacteria will be able to multiply and become part of the soil microflora. The metabolic profile of soil isolates of 48 *E. coli* and 30 enterococci were determined based on their ability to metabolize 95 different carbon sources using the Biolog System. These results showed that many metabolically diverse strains of *E. coli* as well as six different species of *Enterococcus* were recovered from seven different groups of soil in Hawaii. These results support the hypothesis that soil conditions in Hawaii are permissive for the growth of most strains of fecal bacteria. Thus, the genetic capabilities of fecal bacteria are sufficiently robust to overcome all of the ecological constraints in the soil environment and have succeeded in becoming a minor but significant fraction of the soil microflora.

1. INTRODUCTION

Concentrations of fecal indicator bacteria (*E. coli*, enterococci) in environmental waters are used to determine the hygienic quality of water and whether these waters are suitable for recreational uses such as swimming or bathing. In the United States, the recreational water quality standards for fresh waters are less than 126 *E. coli*/100 ml, or 33 enterococci/100 ml [1]. In the application of these water quality standards, two basic assumptions are made. The first assumption is that the only significant sources of these bacteria are feces of human/warm-blooded animals and sewage. The second assumption is that these fecal indicator bacteria do not multiply in the environment. These two assumptions are not applicable for tropical island environments such as Hawaii, Guam and Puerto Rico because in these islands,

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the fecal indicator bacteria are naturally present and multiply under ambient environments such as water, plants and soil [2-8]. As a result, in these tropical islands, these fecal indicator bacteria are consistently present in high concentrations in environmental waters and the source of these fecal indicator is not from sewage contamination, which represents a definite a health risk but from ambient environmental sources, which does not represent a definite health risk.

In Hawaii, considerable evidence have been obtained to show that the fecal indicator bacteria (fecal coliform, *E. coli*, enterococci) are able to multiply in the ambient soil environment [3,5] and as a result, the current USEPA recreational water quality standards are not applicable to Hawaii [9,10]. In this regard, it should be noted that the data to establish these water quality standards were obtained from study sites located in the continental USA, which is the located in the temperate region of the world and are applied to all states and US jurisdictions, including those in the tropical region of the world such as Hawaii, Guam and Puerto Rico. Although sufficient evidence have been accumulated to show that the fecal indicator bacteria have become established in the tropical soil environment of Hawaii, there is no clear explanation as to the mechanism involved in their ability to multiply under ambient conditions. The objective of this paper was to test two hypotheses by which these fecal indicator bacteria can become established populations in the soil environment of Hawaii. The first hypothesis is that the soil environment is unfavorable or restrictive for the growth of most fecal indicator bacteria but a small sub-population of fecal indicator bacteria with metabolic characteristics suitable for growth in the soil will be selected. If this hypothesis is favored, the strains of fecal indicator recovered from soil should be limited to a small number of strains with similar metabolic capacity. The second hypothesis is that the soil environment is permissive and provides sufficient conditions for most of the populations of fecal bacteria in feces to be able to survive and multiply in the soil environment. If this hypothesis is favored, the strains of fecal indicator recovered from soil should be comprised of many different strains with diverse metabolic capacity.

2. MATERIALS AND METHODS

2.1. Study site and recovery of fecal bacteria from soil

Of the 50 US states, Hawaii is unique because it is the only state comprised of islands located in the tropical region of the world and it is the only state where fecal indicator bacteria (*E. coli*, enterococci) used by USEPA to establish recreational water quality standards have been readily recovered from ambient soil environments. The study site is island of Oahu where 80% of the population of the state of Hawaii lives. The general soil map for the island of Oahu [11] was used to select soil sampling sites from the seven groups of soils located throughout the island of Oahu. The Most Probable Number (MPN) Method as described in Standard Methods [12] was the primary method used to recover and enumerate the concentrations of these fecal bacteria from soil samples. To recover *E. coli* from soil samples growth of coliform bacteria in Lauryl Tryptose Broth was passed into EC Broth and incubated at 44.5 °C for 24 hr. Bacterial growth with gas production in this broth was then sub-passaged onto EMB Agar at 37 °C to recover the metallic green sheened colonies. These colonies were confirmed as *E. coli* based on growth and positive MUG reaction on Nutrient Agar with MUG at 37 °C. These isolates were then identified as of *E. coli* using the API 20 E Identification System (bioMerieux Vitek, Inc., Hazelwood, MO). To recover the enterococcus group of bacteria, soil

samples were initially inoculated into Azide Dextrose Broth and incubated up to 48 hr at 35 °C. Bacterial growth in this broth was then sub-passaged onto Pfizer Selective Enterococcus Agar at 35 °C to recover esculin positive colonies. These bacterial colonies were confirmed as belonging to the enterococcus group based on their gram positive stain reaction, negative catalase reaction, positive esculin hydrolysis, their ability to grow in 6.5% NaCl at 35 °C and their production of group D antigen. The purified enterococcus bacteria was then identified to the various species belonging to the genus *Enterococcus* using the API 20 S Identification System.

2.2. Metabolic characterization of soil isolates using the Biolog system

Soil isolates identified as *E. coli* or one of the *Enterococcus* species by the API 20 Identification System were independently analyzed by the Biolog System (Biolog, Inc., Hayward, CA). This system is capable of identifying each isolate and characterizing its metabolic potential by evaluating the isolate's ability to metabolize 95 different carbon sources. This 95-test format can produce as many as 2^{95} different positive and negative metabolic reaction patterns. A MicroLog software (release 3.50) was used to calculate and compare the distances or number of metabolic mismatches for each isolate as compared to the prototype strains stored in the Biolog data base. Based on these data, the isolates were identified to the species level and each isolate was characterized with a specific metabolic profile. These data were then used in a cluster analysis using *mlclust* program (release 3.51), which graphically groups all isolates with similar metabolic profiles and the grouping displayed in a one dimensional dendrogram or displayed as two dimensional clustering patterns.

3. RESULTS

3.1. Analysis of *E. coli* soil isolates

Since the USEPA recreational water quality standard [1] is based on the concentration of *E. coli*, the soil isolation methods to confirm for the presence of *E. coli* as described in Standard Methods [12] were used. A total of 48 confirmed *E. coli* isolates which had been recovered from the seven different soil groups located throughout the island of Oahu were identified as *E. coli* using the API 20 E System. These isolates were then independently analyzed using the Biolog System to characterize the ability of each isolate to metabolize each of the 95 different carbon sources. The metabolic reaction pattern or metabolic profile of each isolate was then compared with the metabolic profile of prototype strains stored in the data base. The soil isolate was then identified to species level based on number of metabolic mismatches or Biolog distance which ranged from 0.266 to 6.841 and had a confidence limit or similarity index (SIM) which ranged from 0.564-0.982. The *mlclust* program in Microlog's software, was then used to compare and to group the isolates based on their metabolic profiles. The first cluster analysis results in a one dimensional dendrogram (Figure 1) which clearly shows a range of metabolic diversity among the 48 *E. coli* strains. A second cluster analysis was performed to plot the data in two dimensions so the clustering patterns of the various metabolic strains are visually much more apparent. The two dimensional cluster analyses of the 48 *E. coli* isolates are plotted in Figure 2 and show that 44 of the 48 strains of *E. coli* clustered into five metabolic groups. The four remaining *E. coli* isolates as well as one ATCC prototype strain of *E. coli* separated into five individual lineages. These results show that the *E.*

coli strains recovered from the soil sites from Oahu are metabolically diverse. These results argue against the first hypothesis that soil environment in Hawaii is very restrictive and will therefore select for a specific population of *E. coli* with specific metabolic properties which will enable them to multiply in the ambient soil environment. These results support the second hypothesis that the ambient soil environment in Hawaii is permissive for the growth of most strains of *E. coli* and therefore most strains of *E. coli* can be expected to grow in the ambient soil environment of Oahu and probably other tropical soil environments.

3.2. Analysis of *Enterococcus* soil isolates

Since the USEPA recreational water quality standard [1] is based on concentrations of enterococci bacteria, the soil isolation methods as described in Standard Method [12] were used to confirm for the presence of enterococci bacteria. A total of 34 enterococci recovered from various groups of soil from Oahu were then identified into six different species of *Enterococcus* (*E. avium*, *E. casseliflavus*, *E. durans*, *E. faecalis*, *E. faecium*, *E. gallinarum*) using the API 20 S System. These isolates were then independently analyzed by the Biolog System. The results summarized in Table 1 show that the identification results with API 20 S generally matched those of Biolog. Thus, all soil isolates identified as *E. faecalis* by API 20 S were also identified as *E. faecalis* by Biolog. However, some of the isolates identified as *E. casseliflavus* by API 20 S System were identified as *E. gallinarum* by the Biolog System and four of the isolates identified as *E. durans* by the API 20 S System were not identified by the Biolog System. These slight discrepancies reflect differences in the measured parameters used for their identification as well as comparison with a different set of control prototype strains. The significance of identifying six species of *Enterococcus* from the soil provide direct evidence that the enterococci bacteria recovered from the soil environment of Hawaii are comprised of metabolically diverse groups. The metabolic profiles of the 30 enterococci isolated from the soil were then subjected to cluster analysis. The results are plotted in two dimensions (Figure 3) and show that metabolic diversity is the basis for grouping into each species. However, metabolic diversity of strains was observed within each *Enterococcus* species. These results provide independent data to support the second hypothesis that Hawaii's soil environment is permissive for the growth of fecal bacteria, including different species of *Enterococcus* bacteria.

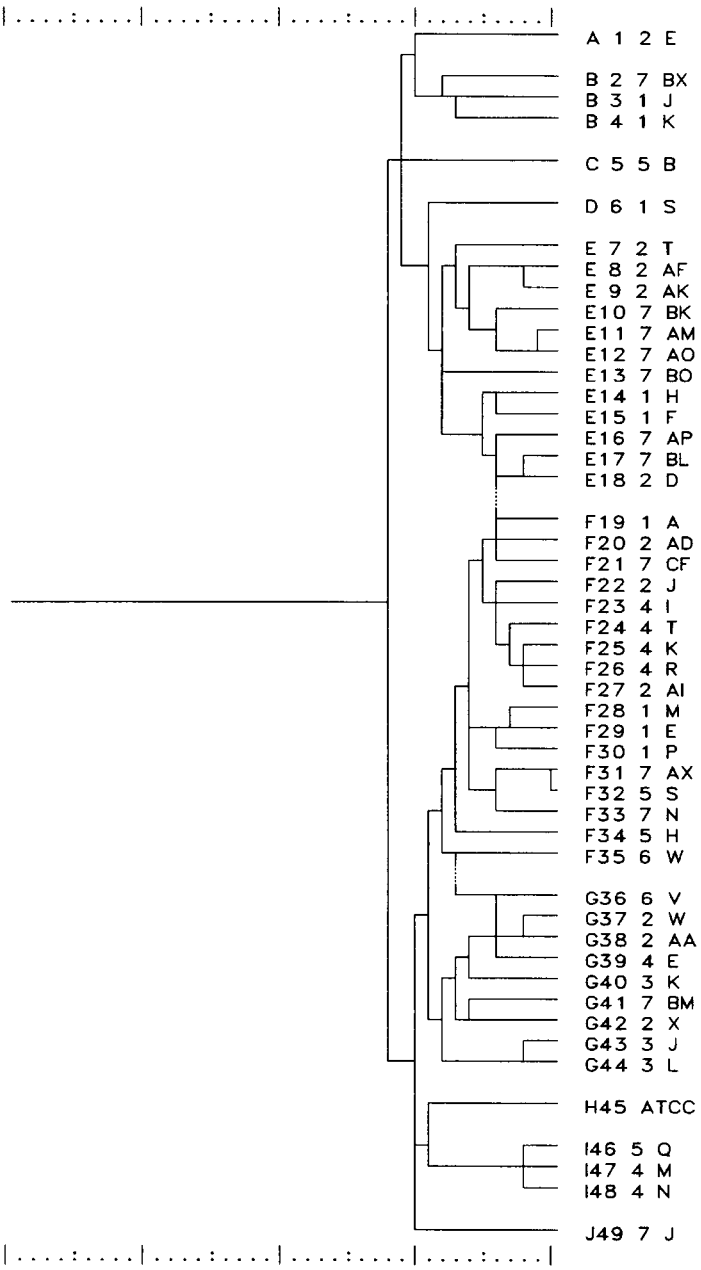
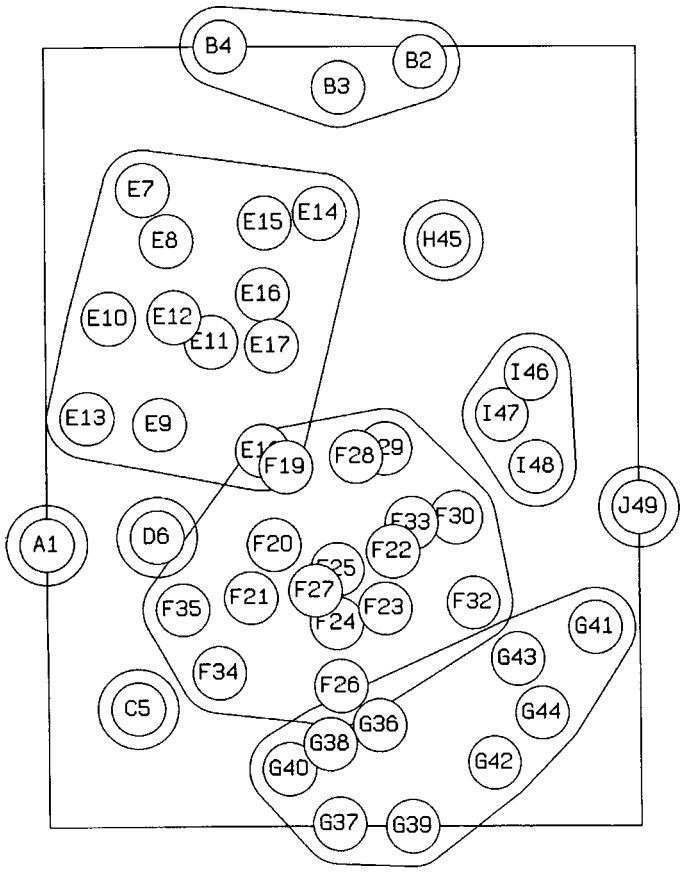


Figure 1. Dendrogram showing the relationships among soil *E. coli* strains (as determined by MicroLog software release 3.51) based on the UPGMA cluster analysis method.



A1 2 E
B2 7 BX
B3 1 J
B4 1 K
C5 5 B
D6 1 S
E7 2 T
E8 2 AF
E9 2 AK
E10 7 BK
E11 7 AM
E12 7 AO
E13 7 BO
E14 1 H
E15 1 F
E16 7 AP
E17 7 BL
E18 2 D
F19 1 A
F20 2 AD
F21 7 CF
F22 2 J
F23 4 I
F24 4 T
F25 4 K
F26 4 R
F27 2 AI
F28 1 M
F29 1 E
F30 1 P
F31 7 AX
F32 5 S
F33 7 N
F34 5 H
F35 6 W
G36 6 V
G37 2 W
G38 2 AA
G39 4 E
G40 3 K
G41 7 BM
G42 2 X
G43 3 J
G44 3 L
H45 ATCC
I46 5 Q
I47 4 M
I48 4 N
J49 7 J

Figure 2. Relationships among *E. coli* strains depicted in a 2-dimensional plot generated by MicroLog software release 3.51.

4. DISCUSSION

The focus of this study was to provide an explanation for the ability of fecal indicator bacteria to multiply and become established as part of the microflora of soil in Hawaii [3,13]. These findings contradict two well established concepts used in the application of water quality standards. The first established concept is that concentrations of these fecal indicator bacteria in environmental waters can be used to determine the extent by which these waters are contaminated with feces or sewage because feces and sewage are accepted as the only significant sources of these fecal indicator bacteria. The second established concept is that since the normal habitat of fecal indicator bacteria (*E. coli*, enterococci) is the intestines of warm blooded animals, these intestinal bacteria should not be able to multiply and become established in the ambient environments such as the soil. In this regard, most scientists believe that these concepts are applicable throughout the world because textbooks used to train scientists in public health microbiology support these two concepts and reports of contradictory evidence from tropical islands such as Hawaii, Guam and Puerto Rico are generally ignored or dismissed as academic studies.

The objective of this study was to test two hypotheses by which these fecal indicator bacteria can become established populations in the soil environment of Hawaii. The first hypothesis states that the soil environment is unfavorable or restrictive for the growth of most fecal indicator bacteria but a small sub-population of these fecal bacteria with metabolic characteristics suitable for growth in this restrictive environment will be selected to establish a population in the soil environment. If this hypothesis is favored, the strains of fecal bacteria recovered from the soil should be restrictive to few strains with similar metabolic capacity. The second hypothesis states that the soil environment of Hawaii is permissive and provides for sufficient conditions to accommodate the metabolic potential of most fecal bacteria to grow in the soil environment of Hawaii. If this hypothesis is favored, the strains of fecal bacteria recovered from the soil should be comprised of many different strains with diverse metabolic capacity. To test these two hypotheses, we tested the ability of soil isolates of *E. coli* and enterococci to metabolize 95 different sources of carbon using the Biolog System. In this regard, Biolog's substrate utilization system has been previously used to identify and assess metabolic variation among bacterial strains from natural environments [14,15]. The results of this analysis showed that the 48 *E. coli* isolates which had been recovered from seven different soil groups on the island of Oahu were comprised of several metabolically diverse strains. Moreover, 34 soil isolates belonging to the general enterococcus group were identified as belonging to metabolically diverse groups comprised of six different species of *Enterococcus* (*E. faecalis*, *E. faecium*, *E. casseliflavus*, *E. gallinarum*, *E. avium*, *E. durans*). These results support the hypothesis that the soil environment of Hawaii is permissive for the growth of fecal indicator bacteria such as *E. coli*, and *Enterococcus* spp.

Since the prevailing concept is that fecal indicator bacteria cannot multiply in ambient soil environments, many water quality managers as well as scientists, especially those in the public health community, will find the conclusions of this current study difficult to believe. However, it should be pointed out that the public health community readily accept the fact that total coliform bacteria, whose normal habitat is the ambient environment, are natural populations in the intestines of warm blooded animals. Thus, it should be reasonable to expect

that fecal indicator bacteria, whose natural habitat is the intestines of warm blooded animals, can establish populations in soil environment along with total coliform bacteria, especially in humid and tropical regions of the world. The question of whether fecal indicator bacteria have the capacity to multiply in tropical soil environment should be viewed from a microbial ecology perspective because microbial populations of the soil environment are controlled by principles of microbial ecology which can be summarized by the general statement that in the environment, the principles of microbial ecology determine which microorganism will become established. In this regard, it is well known that every microorganism will use all of its genetic resources to survive in any given environment [16]. Thus the ability for any microorganism to become established in any environment is based on matching its genetic capacity with the availability and suitability of the various parameters in that environment which affect microbial growth.

In assessing the ability of fecal bacteria (*E. coli*, enterococci) to grow in soil conditions of Hawaii, the important growth controlling parameters should be assessed. Soil temperature in Hawaii generally ranges between 22-30 °C which are well within the growth range of fecal indicator bacteria. Available moisture, sufficient nutrients and favorable conditions for biological growth are characteristics of soil in humid tropical area as exemplified by year long growth of foliage and high density of insects, worms and microorganisms in the soil. Competition for nutrients and predation by other soil microflora are known to control the populations of microorganisms in soil. In this regard, we previously determined that fecal bacteria comprise a minor population as compared with other microbial populations in soil [3]. These results indicate that ambient soil conditions in Hawaii are sub-optimal for the growth of fecal bacteria and are much more favorable for the growth of other groups of microorganisms. Despite these sub-optimal conditions, the establishment of fecal bacteria in the soil environment of Hawaii is significant because this environmental source of fecal indicator bacteria interferes with the usefulness of current water quality standards in Hawaii. However, the principles of microbial ecology states that for fecal bacteria to become an established soil microflora, minimal rather than optimum growth conditions must be met. It is clear that the genetic capacities of fecal bacteria such as *E. coli* and enterococci are sufficiently robust to overcome all of the ecological constraints in the tropical soil environments and they have succeeded in establishing themselves as part of the soil microflora in Hawaii and probably many similar environments in the world.

Table 1
Biolog species identification patterns for enterococci isolates recovered from soil

Soil Isolate No.	Species Identification by API	Biolog ID	Similarity Index	Soil Isolate No.	Species Identification by API	Biolog ID	Biolog Similarity Index
1 B[e]	*Enterococcus casseliflavus*	*E. gallinarum*	0.732	7 G[e]	*E. gallinarum*	*E. gallinarum*	0.713
1 C[e]	*E. casseliflavus*	*E. gallinarum*	0.783	7 H[e]	*E. gallinarum*	*E. gallinarum*	0.800
1 E[e]	*E. gallinarum*	*E. gallinarum*	0.786	7 L[e]	*E. gallinarum*	*E. gallinarum*	0.809
1 G[e]	*E. gallinarum*	*E. gallinarum*	0.809	7 M[e]	*E. gallinarum*	*E. gallinarum*	0.813
1 I[e]	*E. gallinarum*	*E. gallinarum*	0.809	7 V[e]	*E. durans*	No ID	
1 P[e]	*E. casseliflavus*	*E. gallinarum*	0.792	7 X[e]	*E. casseliflavus*	*E. gallinarum*	0.766
1 T[e]	*E. casseliflavus*	*E. gallinarum*	0.698	7 AA[e]	*E. casseliflavus*	*E. gallinarum*	0.744
2 B[e]	*E. casseliflavus*	*E. gallinarum*	0.826	7 AC[e]	*E. avium*	*E. avium*	0.907
2 F[e]	*E. casseliflavus*	*E. gallinarum*	0.752	7 AD[e]	*E. casseliflavus*	*E. gallinarum*	0.768
4 S[e]	*E. casseliflavus*	*E. gallinarum*	0.742	7 AG[e]	*E. casseliflavus*	*E. gallinarum*	0.744
5 A[e]	*E. faecalis*	*E. faecalis*	0.883	7 AI[e]	*E. faecalis*	*E. faecalis*	0.907
5 B[e]	*E. faecalis*	*E. faecalis*	0.872	7 AQ[e]	*E. faecium*	*E. faecium*	0.899
5 C[e]	*E. faecalis*	*E. faecalis*	0.887	7 AT[e]	*E. faecalis*	*E. faecalis*	0.880
5 D[e]	*E. faecalis*	*E. faecalis*	0.922	7 WS 1[e]	*E. durans*	No ID	
5 F[e]	*E. faecalis*	*E. faecalis*	0.887	7 WS 2[e]	*E. durans*	No ID	
6 P[e]	*E. casseliflavus*	*E. gallinarum*	0.818	7 WS 3[e]	*E. durans*	No ID	
7 A[e]	*E. faecalis*	*E. faecalis*	0.959	ATCC 29212	*E. faecalis*	*E. faecalis*	0.949
7 C[e]	*E. faecalis*	*E. faecalis*	0.953	ATCC 19433	*E. faecalis*	*E. faecalis*	0.843

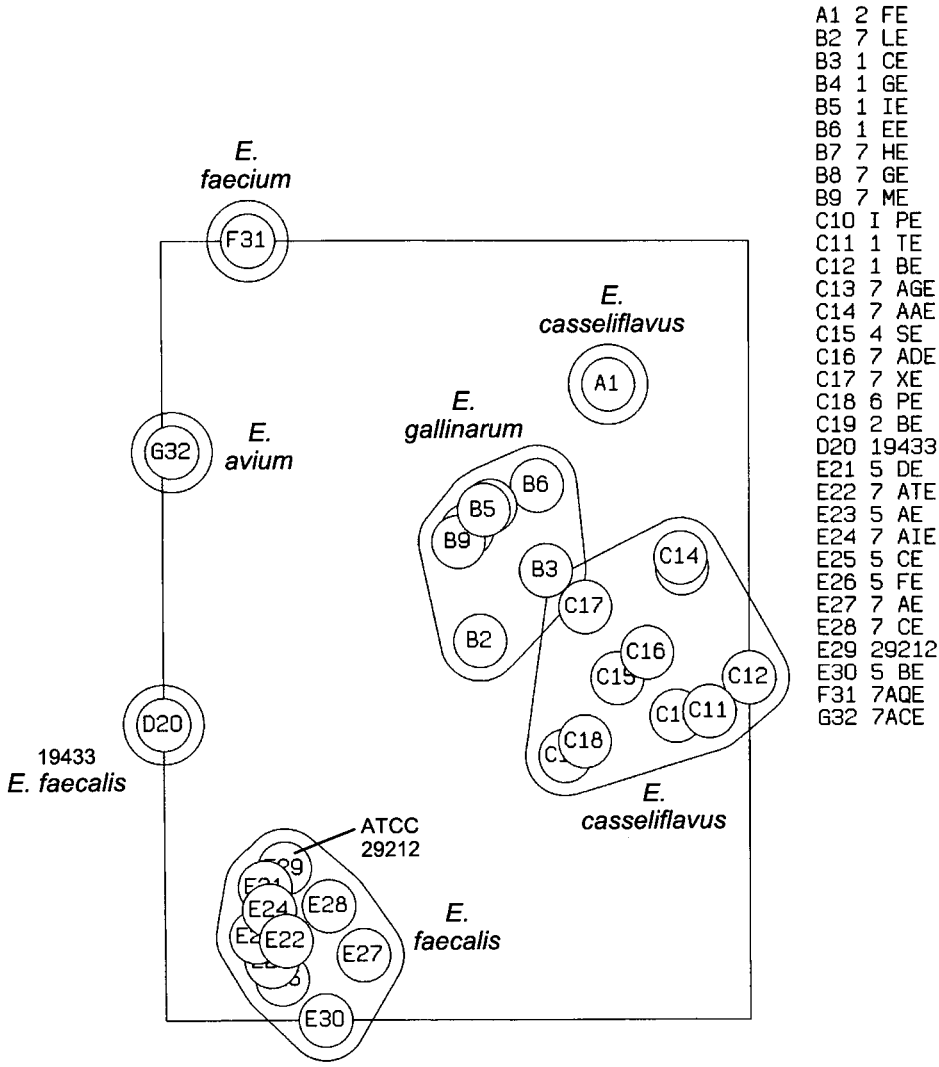


Figure 3. Relationships among soil enterococci isolates as shown in a 2-dimensional plot generated by MicroLog software release 3.51.

REFERENCES

1. A. Dufour, Health Effects Criteria for fresh Recreational Waters, EPA-600/1-84-004 (1984).
2. R.S. Fujioka, K. Tenno, and S. Kansako. Toxicity Assess. 3 (1988) 613.
3. R.S. Fujioka and M.N. Byappanahalli, Water Resources Research Center, University of Hawaii, Proj. Rep. No. WRRC-98-04 (1998) 85.
4. C.M. Hardina and R.S. Fujioka, Environ. Toxicol. Water Qual., 6 (1991) 185.
5. M.N. Byappanahalli and R.S. Fujioka, Water Sci. Tech., 38 (1998) 171.
6. R.S. Fujioka, C. Sian-Denton and K. Morphew, J. Appl. Microbiol. 85 (1999) 83S.
7. M. Bermudez and T.C. Hazen, Appl. Environ. Microbiol. 54 (1988) 979.
8. G.A. Toranzos, Environ. Toxicol. Water Qual. 6 (1991) 121.
9. R.S. Fujioka and M.N. Byappanahalli, Water Resources Research Center, University of Hawaii, Proj. Rep. No. WRRC-96-01 (1996) 50.
10. R. Fujioka, B. Roll and M.N. Byappanahalli, Proc. Water Environ. Fed. 70th Annual Conf., Chicago, Illinois, USA (1997) 405.
11. Soil Survey of Islands of Kauai, Oahu, Maui, Molokai, and Lanai, State of Hawaii. Published by United States Department of Agriculture, Soil Conservation Service in Cooperation with the University of Hawaii Agricultural Experiment Station.
12. Standard Methods for the Examination of Water and Wastewater, American Public Health/American Water Works Association/Water Environment Federation, Washington DC, 18th ed., 1992.
13. M.N. Byappanahalli and R.S. Fujioka. 99th General Meeting of the American Society for Microbiology, Chicago, Illinois, USA (1999).
14. C. Verniere, O. Pruvost, E.L. Civerolo, O. Gambin, J.P. Jacquemoud-Collet and J. Luisetti, Appl. Environ. Microbiol., 59 (1993) 243.
15. A.T. Bernards, L. Dijkshoorn, J. Van Der Toorn, B.R. Bochner and C.P.A. Van Boven, J. Med. Microbiol., 42 (1995) 113.
16. 16. M.R. Atlas and R. Bartha, Microbial Ecology: Fundamentals and Applications, 4th ed., Benjamin/Cummings Publishing Company, Inc., California, USA, 1993.

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Anaerobic wastewater treatment in (sub-)tropical regions

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Over 1200 full-scale anaerobic wastewater treatment plants have been installed worldwide so far, over 40% of which are in the (sub-)tropical regions. Although the pace has picked up rapidly in the past 10 years, the market potential is still enormous. The application of anaerobic treatment process in (sub-)tropical regions has not just been limited to the traditional effluents of agro-industries, but has also been extended to the treatment of effluents from chemical industries, landfill leachate, municipal wastewater, etc.. In this paper, applications in China, India, Taiwan, Thailand, Philippines, Brazil and Mexico are discussed in detail. A few examples: China has installed over 5.7 millions of family-size anaerobic digestors producing biogas used for cooking and lighting by rural farming communities; Mexico has the largest UASB plant in the world (83,700 m^3) treating combined industrial and municipal wastewater; Nearly all the PTA (purified terephthalic acid) plants in Taiwan, the largest producer in the world, treat effluent anaerobically, including one having a total reactor volume of 15,000 m^3; An anaerobic municipal wastewater plant is in design in Brazil for one million inhabitants.

1. INTRODUCTION

Under anaerobic conditions, organic pollutants in wastewater are degraded by microbes, producing methane and carbon dioxide. The degradation process can be highly effective [1]. It produces only 5-10% of sludge as compared to the more conventional aerobic processes [2], and thus saves considerably cost associated with the sludge disposal. In addition, since it does not require aeration, anaerobic process also saves substantial amount of cost associated with aeration, including equipment, maintenance and energy consumption. Besides, the process converts organic matters into methane, a readily usable fuel. The aspects of energy saving/production and minimum maintenance make anaerobic process particularly attractive to the developing countries. Many rural communities in these countries cannot afford the capital and operational expenses for the aerobic treatment process, and some may not even have adequate supply of electricity to run the equipment at all. The process, which favors warm temperature, is especially attractive for the subtropical and tropic regions.

Traditionally, anaerobic process is used for the digestion of livestock wastes and sludge produced by the activated sludge plants. Since the late 1960's, a number of high-rate anaerobic reactors have been introduced for wastewater treatment. Among them, the most popular ones are anaerobic filter (AF) [3], and upflow anaerobic sludge blanket (UASB) [4].

The basic design principle of these reactors is to retain the sludge inside the reactor in order to increase the treatment efficiency. These reactors are highly effective for the degradation of fatty acids [5] and carbohydrates [6], and have been widely applied for the treatment of wastewater from food, brewery, and agricultural industries. More recently, they have also been found effective for the treatment of refractory pollutants, such as proteinaceous matters [7] and aromatic chemicals [8-10]. The application has thus been extended to the treatment of wastewater from chemical industry [11], and municipal wastewater as well [12,13].

This paper is to present an overview of present situation on the implementation of anaerobic technology in full-scale wastewater treatment. The worldwide applications is to be summarized, followed by the discussion of applications in the tropical and sub-tropical regions for industrial, municipal, and rural wastewater treatment. Applications in China, Taiwan, India, Thailand, Philippine, Brazil and Mexico are to be discussed in more details.

2. WORLDWIDE APPLICATIONS

Complete information of worldwide application of anaerobic wastewater treatment is difficult to keep track. Many plants were installed in the last 10 years, and the number is continued to increase each year. A 1990 survey [14] showed that the number of UASB plants alone at that time was 205, but the number had been escalated to 793 by 1998 [15]. In addition, many tropical and subtropical countries are either non-English speaking or under-developed. Relevant information of these countries is often unpublished, or only published in their native languages and thus unknown to the international communities. China has widely applied anaerobic technology for waste/wastewater treatment in rural areas for over two decades. Yet, reliable statistics and information on their experience are hard to find even in Chinese publications. Brazil is believed to have over 200 anaerobic sewage treatment plants built by local firms [16], but yet very little of this information is known elsewhere. On the other hand, getting information from international companies is also not easy. Two cases in point: BP-Amoco has developed a proprietary anaerobic process for the treatment of PTA (purified terephthalic acid) plant effluents; it has kept technology in-house without intention to publicize or commercialize it. As a result, only two of BP-Amoco plants were identified in a 1999 survey [11], while there are at least eight in existence. Furthermore, some companies are reluctant to reveal information, afraid of inviting competitions. In explaining the reason that its company does not publish its installation list, an executive of one leading company wrote to the authors that "..(one competitor).. to go and bother our customers frequently to ask them to change suppliers."

One of the most comprehensive survey in recent years was conducted by the Deutsche Geellschaft fur Technische Zusammenarbeit (GTZ) on behalf of the German Federal Ministry for Economic Cooperation. The resulting report in 1998 [15] showed that over 1229 full-scale plants had been identified worldwide. Japan leading the rest of the world with 162 anaerobic industrial wastewater treatment plants installed, followed by Germany (115), Netherlands (91), USA (83) and India (79). Judging by the "anaerobic reactor density", which was defined as number of plants per million inhabitants, The Netherlands with a value of 5.83 was clearly the leader. This density factor is strongly dependent on the degree of industrialization of individual countries. The values for Mexico and Brazil, the two leading countries in Latin America, were 0.46 and 0.40, respectively, as compared to 0.06 for India, the leading country

Table 1
Reactors, wastewater sources and leading companies in anaerobic treatment [15]

A: Reactor type

Reactor	Number
UASB	793
Anaerobic contactor	112
Anaerobic filter	104
Anaerobic pond	66
IC	50
EGSB	50
Hybrid	33
Fluidized bed	21
Total	1229

B: Wastewater source

Wastewater	Number
Food	336
Beer	207
Domestic	159
Distilleries	136
Pulp/paper	87
Sugar	76
Solid waste	71
Chemicals	71
Manure co-digestion	65
Beverage	58

C: Top ten companies

Company	Number
Paques	312
Biothane	250
Biotim	96
ADI	68
Purac AB	59
Degremont SA	56
Enviroasia	54
Kurita	39
Shinko	28
TBW GmbH	27

UASB: upflow anaerobic sludge blanket
IC: internal circulation
EGSB: expanded granular sludge bed

in Asia. These values clearly show that there is an enormous market potential for anaerobic technology in Latin America and in Asia.

The report also showed that UASB was the most common type of reactor design. With 793 plants, it accounted for 64.5% of the total number of reactors. Anaerobic contactor (112) and anaerobic filter (104) were the second and third most common types of reactors. These two reactors have been on the market for the treatment of agro-industrial wastewater since the early days; however, their growth is not expected to be substantial. Instead, several new reactors are expected to grow in popularity, including those of EGSB (expanded granular sludge bed), internal-circulation and fluidized-bed. Table 1A shows the details of various types of reactor installations. Table 1B summarizes the numbers installations for eight types of industrial effluents, plus solid wastes and co-digestion. Effluent from food industry, with 336 plants, was the most common, followed by brewery (207), sewage (159), distillery (136), pulp/paper (87), etc. Organic matters in the effluent of these industries are mostly easily-degradable carbohydrates. More recently, the technology has been extended to the treatment of effluent from chemical industries [11], including those containing concentrated organic acids, formaldehyde, phenol, aspartame, ethylene glycol, plastics, etc.

Table 1C lists the top ten leading companies in anaerobic wastewater treatment at the end of 1996. The top four companies at that time, i.e. Paques, Biothane, Biotim and ADI, installed a total of 726 plants, representing 57% of the total. A more recent survey conduct for the present study, indicated that the top four companies, i.e. Paques [17], Biothane [18], Enviroasia [19] and ADI [20], installed a total of 773 plants by 1999 treating 13,360 tons of COD daily. Out of these plants, 59 had daily capacity of treating over 50 tons of COD removing a total of 5,152 tons of COD each day.

3. APPLICATIONS IN (SUB-)TROPICAL REGIONS

A survey conducted for the present study in 1999 also showed that 338 of the 773 anaerobic wastewater treatment plants were installed in tropical and subtropical regions by the

four leading international companies. Table 2 shows that a total of 77 plants were installed in Brazil, followed by India (75), China (43), Mexico (22), Philippines (22), Taiwan (21), Thailand (14), etc. The COD removal capacities in these seven regions total 6,418 tons/day. Details of anaerobic wastewater treatment in these seven regions are discussed as follows:

3.1. China

More than 600 anaerobic reactors were built in China to treat wastewaters from poultry and cattle farms, breweries, sugar, and pharmaceutical production plants [21]. These anaerobic reactors had a total volume of 220,000 m^3 and produced biogas to 84,000 families for heating. Among them, the largest anaerobic treatment system, consisting of two 5,000 m^3 anaerobic reactors and a 10,000 m^3 biogas storage tank, was built in 1987 for the Nanyang Distillery in Henan province. It produces 40,000 m^3 of biogas daily for 20,000 households. In a suburban district of Shanghai, thirteen reactors with a total volume of 4,100 m^3 were installed for a large farming community treating animal manure, bean-processing wastewater, and slaughter wastewater [22]. The system produces 4,920 m^3/d of biogas for the consumption of 2,868 farm workers.

By 1995, there were 5.7 million underground anaerobic reactors in rural China treating agricultural and human wastes, animal manure and food residues [23]. Over 200,000 new installations have since been added annually [21]. In many remote rural areas without electricity supply, farmers installed these reactors to produce biogas for cooking and lighting. A reactor treating household wastes and animal manure can provide about 60% of energy needed by a farming family [24]. The total annual biogas production in rural China amounted to over 10^9 m^3 [25]. In Sichuan province alone, 2.2 million of such reactors producing 6.15×10^8 m^3 of biogas annually for 25 million people in rural areas [21,23].

Industrial pollution is extremely severe in China. Many financially struggling plants still discharge extremely high-strength effluents into the environment with limited treatment, including many of sugar, textile, food, pulp/paper plants. Only a small fraction of plants treat their high-strength wastewater using anaerobic process, many of which are from distillery,

Table 2
Anaerobic treatment plants in (sub-) tropical regions installed by four international companies

Country	Number	COD removal Ton-COD/d	Country	Number	COD removal Ton-COD/d
Brazil	77	1046.2	South Africa	3	41.0
India	75	3377.9	Argentina	2	30.9
China	43	366.5	Vietnam	2	17.0
Mexico	22	413.4	Hong Kong	1	12.4
Philippine	22	454.2	Guatemala	1	50.4
Taiwan	21	207.5	Malawi	1	1.1
Thailand	14	552.5	Kenya	1	100.8
Indonesia	10	31.8	Mauritius Island	1	0.8
Colombia	9	146.3	Nepal	1	49.0
Israel	9	67.8	Pakistan	1	756.0
Venezuela	9	264.1	Puerto Rico	1	1.5
Malaysia	8	142.2	Saudi Arabia	1	10.0
Chile	3	2.7	Singapore	1	12.5
			Total	338	7476.1

winery, pharmaceutical and chemical industries. Three of the top four international companies, Paques, Biothane and Enviroasia, entered the Chinese market in 1994; ADI remains absent as of today. By 1999, these three companies have installed a total of 43 anaerobic plants treating 366 tons of COD daily. Among the 43 plants, 17 are for beverage effluent, 18 for brewery, 3 each for food and potato, and 1 each for coffee and chemical. UASB is the most common type of reactor, representing 86% of all of these reactors. Anaerobic treatment plants in China are small. Twelve of these 43 plants have the daily COD removal capacity of less than 5 tons, and the largest one has the capacity of only 36.75 tons/d. A 1998 survey indicated that over 30 anaerobic plants had been installed by local companies in China treating industrial wastewater [26]. Seven of which use UASB type of reactors. Twelve of the 30 plants have reactors over 100 m^3 in volume, including five in the range of 1,000-3,600 m^3. Most of the plants treat high strength effluent with COD over 10,000 mg/l. Very few brewery effluents in China is treated anaerobically. It is often combined with bottle-washing and sanitary effluents, and the final effluent has less than 1,000 mg/l of COD [27].

Anaerobic treatment of domestic wastewater is mainly applied in the form of septic systems. All domestic wastewater is firstly discharged into small septic tanks near each housing complex. Solids are hydrolyzed and partially degraded in the septic tanks, and only the supernatant is discharged into municipal sewerage. Sludge built up in the tank is removed every 1-2 years. Biogas generated in the urban septic tanks are mostly vented. However, in the rural areas in southern China, biogas generated from these septic tanks are recovered for utilization. Zhang *et al.* [21] estimated that 35,700 biogas-recovering septic systems had been built by 1995 treating the domestic wastewater of 2 million population and supplying biogas as fuel for 22,000 households. A septic tank has a typical volume of 100-200 m^3 treating 30-70 m^3/d of domestic wastewater. Typical design parameters are 3 days of HRT, 0.2 kg-COD/m^3/d of loading, and 0.05 $m^3/m^3/d$ of biogas production rate [28].

3.2. India

In 1996, 3,674 tons of COD were removed daily by anaerobic processes in 79 industrial and 20 municipal wastewater treatment plants [15]. Such a COD removal capacity was far higher than any other countries in the world; for comparison, USA, the runner-up, only removed 2,019 tons of COD daily. Local companies designed and built most of municipal wastewater treatment plants in India, whereas international companies built the industrial wastewater treatment plants. Compared to those in other tropical/subtropical countries, anaerobic reactors in India are large and are used widely for the treatment of distillery effluent. The four leading companies, Paques, Biothane, Enviroasia and ADI, alone have installed a total of 75 anaerobic plants in India since 1986. Of these plants, 31 are over 5,000 m^3 in reactor volume, and 32 are in the range of 1,000-5,000 m^3; only 11 are smaller than 1000 m^3, accounting for only 1% of total COD capacity. All but one of these 75 plants are treating industrial wastewater, including 44 for distillery, 8 for chemical, 6 for pharmaceutical, 5 for paper, 3 each for brewery and starch, 2 for food and 1 each for dairy, textile and combined effluent. Forty plants treat distillery wastewater, accounting for 83% of the total COD removal capacity.

UASB is the most common type of reactor in India, totaling 47 in number and treating 53% of COD removal capacity. The second popular type of reactor is ADI's BVF®, a low-rate covered anaerobic pond system, accounting for 76% of total reactor volume and 44% of total COD removal capacity. Among the 23 BVF® reactors, 19 are treating distillery wastewater

with an average COD of 98,000 mg/l. These reactors are all huge. The average size of the 19 distillery-treating reactors is 22,140 m^3, producing 510,855 m^3 of biogas daily.

3.3. Philippines

At least 22 anaerobic industrial treatment plants have been installed since 1991, 21 by Enviroasia and 1 by Paques. Among them, 9 were for food industry, 5 for brewery, 3 each for distillery and starch, and 2 for beverage. Sixteen of them use UASB reactors, removing a total of 130 tons of COD daily (29% of the total). The other six plants use UAC reactors, removing 323 tons of COD daily (71% of the total). The sizes of these plants are over a wide range. Eleven of the 22 plants are less than 5 ton-COD/d in capacity, 4 are in the range of 5-10 ton-COD/d, 5 in the range of 10-50 ton-COD/d, and only two are over 50 ton-COD/d. The fractions of the total COD removed by individual groups are 8%, 5%, 25% and 62%.

3.4. Taiwan

The first anaerobic reactor treating industrial wastewater in Taiwan was built in 1984. By 1992, local companies installed 12 anaerobic plants with a total reactor volume of 15,500 m^3, 11 of which use UASB type [29]. All are treating industrial effluents, 7 for winery, 2 for chemical, and 1 each for citric, food processing, and yeast. Since 1992, anaerobic technology has also been applied to the treatment of wastewater from paper [30], dairy [31] and petrochemical industries [11].

International companies, including Paques, Enviroasia, ADI, BP-Amoco, Grontmij, and Proserpol, installed at least 24 anaerobic treatment plants in Taiwan, 23 during 1990-95 but only one thereafter in 1998. These installations treat effluents from a wide range of industrial effluents, including 7 for food, 6 for leachate, 5 for chemical, 2 for brewery, and 1 each for beverage, distillery and paper, plus one for an unspecified mixed effluent. The number of leachate treatment plants (6) is probably the highest in the world. Also, Taiwan, as the largest PTA producer in the world, has widely used anaerobic processes to treat this particular wastewater, including three 5,000 m^3 downflow anaerobic filters with circulation by BP-Amoco, one 7000 m^3 UASB reactor by Grontmij, and two hybrid reactor by ADI (5000 m^3 and 4000 m^3 each) [11]. UASB is the most common type of reactor, 21 in number and treating 68% of COD removal capacity. There are 13 plants having COD removal capacity of less than 5 ton-COD/d, representing 54% of total anaerobic plant number but 8% of total COD removal capacity. However, the three plants treating PTA wastewater account 52% of total COD removal capacity.

Anaerobic technology is also extensively used in Taiwan for the treatment of pig wastes. In Taiwan, the number of pigs increased from 2.9 million in 1970 to 10 million in 1990 [32], and to 12 million by 1999. Only half are for local consumption and the other 50% for export. All pig farms are equipped with wastewater treatment system. In 1994, pig farms in Taiwan produced annually 91,848,162 m^3 of wastewater with 936,851 ton-COD [33]. All of these effluents are treated by anaerobic processes.

3.5. Thailand

A total of 5,500 anaerobic reactors, mostly 4-6 m^3 in volume, had been built prior to 1988 as a result of a campaign to clean up the environment and to provide energy for many small farms [34]. However, only 40% of these reactors were still in operation by 1999; the other 60% had been shut down due to technical problem [34]. Newly installed anaerobic reactors are mainly for the treatment of pig farm effluent. The pig population in Thailand is 8.6 million,

producing 180,000 m^3 of effluent and 2,700 ton of COD daily. The Biogas Advisory Unit (BAU)/Chiang Mai University supported by the government under the National Energy Policy Office (NEPO) started in 1995 to build treatment system for wastes produced by pig farms. A typical system was composed of a low-rate anaerobic channel digester and a UASB reactor, plus an oxidation/stabilization pond used as post-treatment. By 1999, 17 of such systems with a total reactor volume of 42,000 m^3 had been built for farms of 210,000 pigs (2.44% of total pig population in Thailand). It produced 210,000 m^3 of biogas daily, generating 25,200 kw-h/day of electrical energy, and 42,000 kg/d of bio-fertilizer.

Three international companies, Biothane, Enviroasia and ADI, have built 14 anaerobic industrial wastewater treatment plants in Thailand by 1999. Most of them (11) were built during 1991-1996, and only two were built thereafter in 1999. All are treating agro-industrial wastewater, except one treating effluent from a synthetic fiber plant. One UAC/AC plant installed by Enviroasia alone has the capacity of removing 274,000 kg-COD/d from a distillery wastewater, equivalent to 49.5% of the total COD removal capacity. Among the other reactors, 12 are UASB and one is ADI's BVF® reactor.

There are 19 anaerobic industrial wastewater treatment plants built by local companies. Of which, 12 were the result of government-sponsored dissemination of UASB biogas works in the alcohol producing plants by 1996 [35]. Produced methane from these anaerobic reactors was not utilized. Anaerobic treatment of domestic wastewater is limited to septic tanks.

3.6. Brazil

Borzacconi, *et al.* estimated [36] that a total of 396 anaerobic reactors with 3.944×10^5 m^3 in volume, had been installed in Latin America by mid-1994. Among them, 1.823×10^5 m^3 (43%), was for the treatment of industrial wastewater, 97% of which was for the effluent of agro-industry. Of the 168 reactors treating industrial effluent, 138 were UASB representing 82% of the total reactor volume. The most common application was for the treatment of effluents from brewery (41), followed by dairy (24), distillery (15), slaughterhouse (15), beverage (12), yeast (10), coffee (8), etc.. Brazil, having 115 anaerobic plants with a total volume of 117,054 m^3, is the leading country in the Latin America.

The survey conducted for this study shows that Paques, Biothane and Enviroasia alone have built 76 industrial wastewater treatment plants, plus one for sewage treatment, in Brazil since 1984. Among all installations, 50 were for brewery, 5 each for beverage and yeast, 4 for food, 3 each for distillery and starch, 2 for cigarettes, and 1 each for chemical, dairy, paper and pharmaceutical. Brewery effluent alone accounts for 72% of the total COD removal capacity. Among the 76 plants, 59 are UASB reactors, 16 are IC reactors and 2 are EGSB reactors. Plants built by these international companies are often quite large; 38 plants are able to remove 10,000-50,000 kg-COD daily, representing 76% of the total capacity.

Campos [16] estimated that there are over 200 anaerobic municipal wastewater plants in Brazil. This is substantially higher than the 25 estimated in 1998 by Hulshoff Pol *et al.* [15]. The discrepancy is likely due to that most of these plants were designed and built by the local companies and thus unknown by the foreigners. Three plants in Belim, Para, alone are treating sewage from 130,000 inhabitants. Since Brazil has very positive experience in anaerobic municipal wastewater treatment, a number of large scale systems presently are in design [16]. These include a UASB plant in the state of Parawa for one million inhabitants, another UASB plant in Rio de Janeiro treating 4 m^3/s of flow, four systems in Sao Paulo State for 350,000, 140,000, 140,000, and 76,000 inhabitants, respectively.

3.7. Mexico

A survey by Monroy *et al.* [37] shows that in Mexico 85 anaerobic wastewater treatment plants have been installed since the technology was first applied in 1987. The total installed volume is 228,551 m^3 and are treating 216,295 m^3/d and 590 tons of COD daily. UASB reactors account for 74% of the installed volume and local companies have supplied 76% to the anaerobic market.

Mexico has 282 industrial wastewater treatment plants, treating only 12% of the total effluent nationwide. Of which, 43 are anaerobic wastewater treatment plants with a total reactor volume of 93,490 m^3 treating 76,644 m^3 of wastewater daily, accounting for only 0.53% of all industrial effluent. Eight more are under construction for 44,010 m^3 of additional volume, including a single 20,808 m^3 reactor treating PTA wastewater. Most of the wastewaters treated are those containing high levels of easily biodegradable substrates. Plant wastewaters from brewery, coffee, beverage, and yeast, respectively, represent 21%, 14%, 12%, and 5% by number, and 27%, 1%, 2%, and 53% by volume of the 43 plants in operation. Although the main polluting industries in Mexico are sugar, paper, chemical, petrochemical and oil refinery, only four plants among them use anaerobic treatment processes, three for paper and one for petrochemical (dimethylterephthalate).

UASB reactors are used in 58% of industrial anaerobic treatment plants, treating 91% of wastewater by volume. Two plants, using low-rate anaerobic ponds, have 52,500 m^3 of reactor volume but only treat 3,900 m^3 of wastewater daily, representing only 5.1% of total anaerobically treated volume. For the 43 plants, the reactor temperature ranges from 17 to 40°C, with an average of 28.6°C. About 53% of reactors are operated over 30°C, 35% at 20-30°C, and only 12% at 17-20°C. The wastewater COD varies from 700 mg/l to 23,000 mg/l, averaging 5,562 mg/l. HRT also varies widely from 5 to 336 hours, the average being 50 hours. Organic loading ranges from 0.45 to 15 kg-COD/m^3/d, averaging 6.09 kg-COD/m^3/d. All but three plants have COD removal efficiency over 70%, the average COD removal efficiency being 81%. Only 14.6% of anaerobic industrial wastewater treatment plants recover methane for uses, 43.9% flare the biogas and the rest simply vent the biogas into atmosphere. All anaerobic reactors built by international companies have biogas handling system; biogas is either flared or recovered for utilization.

A total of 33 anaerobic municipal wastewater plants were designed and built by local companies, plus one built by a Cuban company (CENIC). The majority of these plants (31) use the UASB design. The volume of these UASB reactors totals 90,697 m^3, representing for 99.7% of all anaerobic municipal treatment plants. One is the largest UASB reactor built in the world treating combined municipal and industrial wastewater; it alone has a volume of 83,700 m^3, and is expected to expand to 133,920 m^3 in the future. The reactor temperature ranges from 10 to 32°C; most reactors are operated around 20°C. The municipal wastewater COD ranges from 213 to 2400 mg/l, averaging 518 mg/l. The HRT ranges from 6 to 30 hours, with an average of 11.6hours. Organic loading ranges from 0.28 to 3.0 kg-COD/m^3/d, averaging 1.25 kg-COD/m^3/d. The COD removal efficiency ranges from 50% to 95%, averaging 65%. The effluent COD ranges from 26 to 480 mg/l, averaging 160 mg/l. Effluents of 25 plants (74%) have COD exceeding 100 mg/l of COD. Most of these plants have post-treatment system, either biological (aerobic lagoon, aerobic and anoxic reactors) or physical (secondary settler, sand filter, and activated carbon). Many effluents are chlorinated before final disposal. All plants vent biogas directly without even removing hydrogen sulfide.

4. CONCLUSION

Over 1200 full-scale anaerobic plants have been installed worldwide so far, over 40% of which are in the (sub-)tropical regions. Although the pace has picked up rapidly in the past 10 years, the market potential is still enormous. The application of anaerobic treatment process in (sub-)tropical regions has not just been limited to the traditional effluents of agro-industries, but has also been extended to the treatment of effluents from chemical industries, landfill leachate, municipal wastewater, etc.. International companies have played an important role in introducing the technology to the (sub-)tropical regions, but some local companies have shown the capacity to compete.

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REFERECES

1. H.H.P. Fang, and H.K. Chui, J. Environ. Eng., ASCE, 119 (1993) 103.
2. Metcalf & Eddy, Inc., Wastewater Engineering, Treatment, Disposal, Reuse, Third Edition, McGraw-Hill, Inc., NY, (1991) 394 and 426.
3. J.C. Young, and P. L. McCarty, JWPCF, 41 (1969) R160.
4. G. Lettinga, A.F.M. van Velsen, S.W. Hobma, W.J. de Zeeuw, and A. Klapwijk, Biotech. Bioengrg., 22 (1980) 699.
5. H.H.P. Fang, Y.Y. Li, and H.K. Chui, J. Environ. Engrg., ASCE, 121 (1995) 153.
6. H.H.P. Fang and T.S. Kwong, Environ. Technol., 16 (1995) 13.
7. H.H.P. Fang, and D.W.C. Chung, Wat. Sci. Technol., 40 (1999) 77.
8. Y.Y. Li, H.H.P. Fang, H.K. Chui, and T. Chen, J. Environ. Eng., ASCE, 121 (1995) 748.
9. H.H.P. Fang., T. Chen, Y.Y. Li, and H.K. Chui, Wat. Res., 30 (1996) 1353.
10. G.M. Zhou, and H.H.P. Fang, Bioresource Technol., 61 (1997) 47.
11. H. Macarie, Proc. of Waste Mnimisation and End of Pipe Treat. in Chem. and Petrochem. Ind., (1999) 405.
12. A.C. van Haandel, and G. Lettinga, Anaerobic Sewage Treatment, John Wiley & Sons Ltd., England, (1994) 33.
13. L. Seghezzo, G. Zeeman, J.B. van Lier, H.V.M. Hamelers, G. Lettinga, Bioresource Technol., 65 (1998) 175.
14. G. Lettinga and L. Hulshoff Pol, Wat. Sci. Technol., 24(8) (1991) 87.
15. L. Hulshoff Pol, H. Euler, S. Schroth, T. Wittur, and D. Grohganz, Proc. of 5th Latin-American Seminar on Anaerobic Wastewater Treat., Oct. 27-30, Vina del Mar, Chili, (1998) .
16. J.R. Campos, (1999) Personal communication.
17. C. Frijters, (1999) Personal communication.
18. Biothane, (1999) http://www.biothane.com

19. J.P. Ombregt, (1999) Personal communication.
20. R.C. Landine, (1999) Personal Communication.
21. A.R. Zhang, L.B. Wu, S.D. Wen, L. Pei, Proc. of 8th Intl. Conf. on Anaerobic Digestion, May 25-29, Sendai, Japan, Vol. 2 (1997) 263.
22. B.N. Zeng, Y. Jin, L.B. Wu, Proc. of 8th Intl. Conf. on Anaerobic Digestion, May 25-29, Sendai, Japan, Vol. 3 (1997) 444.
23. D.Y. Long, B. Pu, S.P. Tang, L.B. Wu, L. Pei, Proc. of 8th Intl. Conf. on Anaerobic Digestion, May 25-29, Sendai, Japan, Vol. 3 (1997) 496.
24. J.P. Henderson, Biocycle, 38 (1997) 79.
25. Y. Liu, R.D. Hu, and F.Q. Li, Proc. of 8th Intl. Conf. on Anaerobic Digestion, May 25-29, Sendai, Japan, Vol. 3 (1997) 200.
26. Y.L. Huo, Chinese Light Industry Press, Beijing, China, (1998) 304. (In Chinese)
27. H.H.P. Fang, J. Zhu, and G. Liu, Biotech. Letters, 11 (1989) 673.
28. L.B. Wu, D.Y. Long, B. Pu, S.P. Tang, Proc. of 8th Intl. Conf. on Anaerobic Digestion, May 25-29, Sendai, Japan, Vol. 3 (1997) 492.
29. S.S. Cheng, Proc. of the 1992 Mainland-Taiwan Environ. Technol. Seminar, Sept. 23-28, Shanghai, China, (1992) 84. (In Chinese)
30. H.S. Liao, C.H. Li, J.R. Liu, G.J. Chai, and S.C. Liao, Proc. 21st Wastewater Treat. Technol. Conf. in Republic of China, Taiwan, Nov. 23-24, (1996) 420. (In Chinese)
31. C.H. Chang, G.J. Tsai, J.R. Liu, H.S. Liao, Proc. 20th Wastewater Treat. Technol. Conf. in Republic of China, Taiwan, Nov. 18-19, (1995) 4-19. (In Chinese)
32. J.S. Chiu, Proc. of the 1992 Mainland-Taiwan Environ. Technol. Seminar, Sept. 23-28, Shanghai, China, (1992) 143. (In Chinese)
33. H.C. Soong, K.R. Lin, W.F. Yang, Proc. of the 1996 Mainland-Taiwan Environ. Technol. Seminar, Dec. 17-19, Chung-li, Taiwan, (1996) 701. (In Chinese)
34. BAU, (1999) http://www.chmai.loxinfor.co.th/-bau/history.html.
35. GTZ, (1999) http://www.gtz.de.
36. L. Borzacconi, I. Lopez, and M. Vinas, Wat. Sci. Tech., 33 (1995) 105.
37. O. Monroy, G. Fama, M. Meraz, L. Montoya, , and H. Macarie, Wat. Res., 34(6) (2000) 1803.

Optimization of enhanced biological wastewater treatment processes using a step-feed approach

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Nitrogen (N) and phosphorus (P) removal efficiency in an enhanced biological nutrient removal (EBNR) process was optimized by compartment reconfiguration and step feed. This process with an anaerobic zone, an aerobic zone, an anoxic zone and an aerobic zone in sequence (AOAO process) exhibited excellent removal efficiency in carbon (C), P, but not in N. By further reconfiguring the AOAO process with an anaerobic zone followed by multiple phases of aerobic and anoxic zones and using a stepwise-feeding mode in anoxic zones (multiple stages EBNR process), effective C, N, and P removal efficiency at 96.5, 76.4 and 100%, respectively, was achieved. The synthetic wastewater fed into multiple stages EBNR process was divided into three parts (Q_1, Q_2 and Q_3). The first initial wastewater flow (Q_1) supplying nutrients for microorganisms growth and phosphorus release was fed into the anaerobic tank while the second flow (Q_2) and the third flow (Q_3) providing external carbon source for denitrification were input into the anoxic zones. By varying the step feed ratio of $Q_1 : Q_2 : Q_3$, different water quality and nutrients concentration in the effluent was obtained. A mathematical model based on Activated Sludge Model No.2d was adopted to simulate the water quality in these two EBNR processes. Results showed a good correlation between the experimental data and the simulated values. The mathematical model could further simulate the performance of the full-scale wastewater treatment plant under different operational conditions.

1. INTRODUCTION

Because the stringent nutrient levels being required in the effluents to protect lakes and other natural water from eutrophication, removal of nutrients from discharged wastewater has been required in many wastewater treatment plants. Most of these biological nutrient removal (BNR) processes including A_2O, MUCT and modified Bardenpho processes consist of a sequential anaerobic and aerobic stage for biological phosphorus removal, and recycle nitrified liquor into anoxic zones to promote the removal efficiency of total nitrogen (T-N) [1]. This approach will require additional energy for liquid circulation and addition of external carbon substrate for denitrification in anoxic zones [2]. Further due to the growth of autotrophic nitrifying organisms in the aerobic tank, external addition of alkaline source is necessary to neutralize pH [3]. As the result, the operational cost of these processes will increase significantly.

In this study, the above shortcomings were improved by reconfiguring BNR process without internal nitrified liquor circulation. This was done by configuring the process into (1) anaerobic, oxic, anoxic, oxic zones in sequence (AOAO process) or (2) an anaerobic zone followed by multiple stages of aerobic-anoxic zones (multiple stages EBNR process). In the second approach, a step feed strategy was used to direct the influent into the first anaerobic zone (Q_1) and to the other anoxic zones (Q_2, Q_3) as an external carbon source for denitrification. The optimal feeding ratio of $Q_1 : Q_2 : Q_3$ for achieving the better N/P removal efficiency was empirically determining by using a pilot-scale EBNR reactor.

To further understand the bacterial conversion and transport processes, the IAWQ Task Group has proposed the Activated Sludge Model No.2d for simultaneous removal of N and P (i.e., nitrification, denitrification and biological phosphorus removal) [4]. Since the kinetics and stoichiometry for these metabolisms are not fully understood in the aforementioned two configurations, the ASM2d was modified and used to quantitatively evaluate these processes. The parameters monitored included the soluble components such as soluble chemical oxygen demand (COD_s), ammonium plus ammonia nitrogen (S_{NH4}), nitrate plus nitrite nitrogen (S_{NO3}) and ortho-phosphates (S_{PO4}) [5].

2. MATERIAL AND METHODS

Figure 1 schematically depicts the AOAO pilot plant. The net volume for individual reactors and settlement tank were 28 liters (anaerobic zone), 70 liters (aerobic zone), 35 liters (anoxic zone), 7 liters (aerobic zone), and 21 liters (settlement zone), respectively. The first initial synthetic wastewater flow (Q_1) supplying nutrients for microorganisms growth and carbon for phosphorus release was fed into the anaerobic tank while the second flow (Q_2) providing external carbon source for microorganisms growth and denitrification was fed into the anoxic zone by stepwise feeding. In addition, the total flow rate (Q_1+Q_2) was maintained at 230 ml/min.

Figure 2 shows the configuration of the multiple stages EBNR process. It consists of an anaerobic zone followed by multiple phases of aerobic and anoxic zones in sequence and using a step-feeding mode in anoxic zones. The volume was 7 liters for the last aerobic zone, and 13.8 liters for the rest reactor zones. Total reactor volume was 131.3 liters. The first initial synthetic wastewater flow (Q_1) was fed into the anaerobic tank. The second flow (Q_2) combined with the third flow (Q_3) was fed into the anoxic zone by stepwise feeding. To improve the T-N removal efficiency, external carbon source (Q_4) such as methanol or acetate was added at the last anoxic zone. The total flow rate (Q_1+Q_2+Q_3) was maintained at 240 ml/min.

The final clarifier included a rectangular settlement area and a cone for sludge thickening. The height of the rectangular settlement zone, volume of the cone and final clarifier, overflow rate and surface loading are 20 cm, 8.3 liters, 56.3 liters, 1.728 m^3/mday and 2.16m^3/m^2day, respectively.

The synthetic wastewater used simulated the effluent nutrients concentration of primary clarifier in a local wastewater treatment plant in Taipei. The composition included full-fat dry milk powder and some laboratory grade chemical compounds, and was summarized in Tables 1 and 2. The analytical steps and principals in this research were performed according to the procedure described in Standard Methods [6].

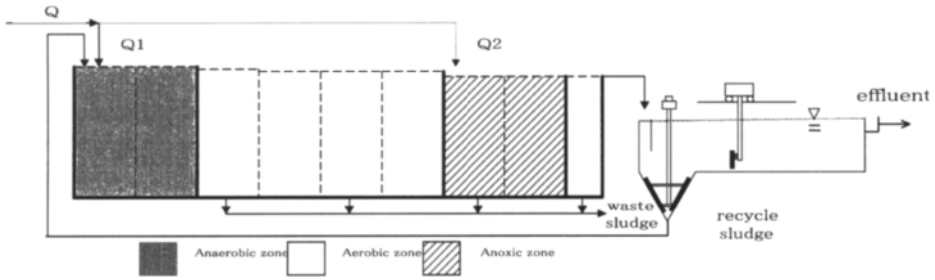

Figure 1. Pilot plant diagram of AOAO process

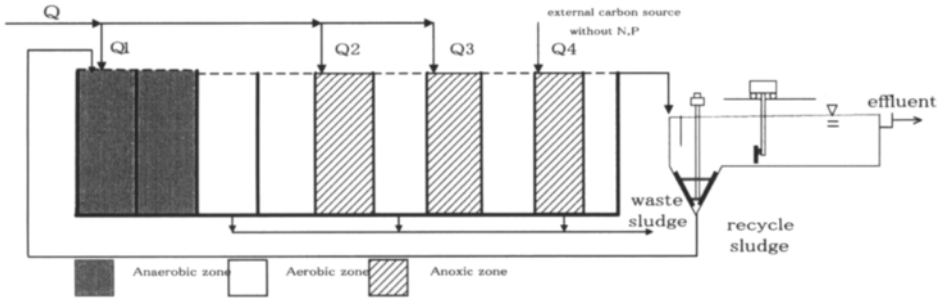

Figure 2. Pilot plant diagram of multiple stages EBNR process

Under the same influent wastewater concentration, these two pilot plants were operated at a recycling sludge ratio of 0.5 without internal recycle of nitrified supernatant. During the operation, the sludge retention time (SRT) was maintained at 10 days, and temperature was controlled at 20 centigrade. A mixer was installed in each reactor to prevent the activated sludge from settling. The residual dissolved oxygen concentration was maintained above 2.5 mg/L in the aerobic tank and above 4.0 mg/L in the final aerobic reactor. Moreover, pH control instrument was set in the first aerobic tank to keep pH value at 7.3. The step feed ratios as shown in Table 3 were used for these two EBNR processes.

The stoichiometric and kinetic parameters were originated from ASM2d. The alkalinity was always higher than the alkalinity saturation coefficient, and was not taken into consideration in the kinetic equations. Because the hydrolysis of organic nitrogen (Org-N) in influent was not included in ASM2d, the kinetic hydrolysis of Org-N proposed in ASM1 was employed to simulate the behavior of Org-N [7]. The effect of biosorption as an important factor was simulated in the model. The equation proposed by Fujie *et al.* for COD_s biosorption in the activated sludge tank was used for calculation [8]. The experimental data from the optimal step feed ratio (0.8 : 0.2) in AOAO process would be compared with simulation values. So would be the multiple stages EBNR process (0.7 : 0.2 : 0.1).

Table 1
Synthetic wastewater components (5 liters)

Components	Weight or volume
$FeCl_{3(aq)}$	5 mL
$NH_4Cl_{(s)}$	200 g
$Urea_{(s)}$	150 g
$KH_2PO_{4(s)}$	75 g
$CH_3COOH_{(l)}$	145 ml
$C_6H_{12}O_{6(s)}$	67.5 g
Full-fat milk $powder_{(s)}$	680 g

Table 2
Characteristics of synthetic substrate

Components	Approximate concentration
total COD(COD_t)	300 mg/L
soluble COD(COD_s)	210 mg/L
suspend solid(SS)	50 mg/L
total Kjeldahl nitrogen(TKN)	40 mg/L
ammonia nitrogen(NH_4^+-N)	20 mg/L
total phosphorus(T-P)	5 mg/L
ortho-phosphorus(ortho-P)	4 mg/L
alkalinity(as $CaCO_3$)	60 mg/L
pH	7

Table 3
Experimental runs in EBNR processes

Run	AOAO Process	Multiple Stages EBNR Process
	$Q_1 : Q_2$	$Q_1 : Q_2 : Q_3$
No. 1	0.7 : 0.3	0.7 : 0.2 : 0.1
No. 2	0.8 : 0.2	0.8 : 0.1 : 0.1
No. 3	1 : 0	1 : 0 : 0

3. RESULTS AND DISCUSSION

3.1 AOAO process

In these three testing runs, the effective removal efficiency of carbon, total phosphorus (T-P) and total nitrogen (T-N) at 90~95, 95~100 and 50~70%, respectively, were achieved. The optimal effluent water quality with the least T-N concentration was obtained while the feed ratio was 0.8 : 0.2 (run 2), and the average mixed liquid suspended solid (MLSS) in this run was equal to 1800~2000 mg/L.

Table 4 summarizes the residual nutrients concentration in each stage. The result indicates that the removal efficiency of COD, T-P and T-N were 92.3, 100 and 70.0%, respectively.

The profiles of the nutrient concentration under the optimal run were shown in Figures 3-6, and the experimental results were further compared with those from the simulated values based on ASM2d model.

Incomplete COD_s removal observed in Figure 3 was possibly caused by the un-biodegradable substrate in the synthetic wastewater. Meanwhile, residual COD_s revealed no significant change in the system besides the first anaerobic tank. When the step feed ratio (Q_1 : Q_2) was increased from 0.7 : 0.3 to 1 : 0, nitrate became as the predominant nutrient in the effluent. On the other hand, the major N-containing nutrients were ammonium and organic nitrogen at a feed ratio lower than 0.7 : 0.3. Further comparison between experimental data and simulated results would be discussed later.

Table 4
Steady-state experimental results in the AOAO process under $Q_1 : Q_2 = 0.8 : 0.2$

Nutrients	COD_s (mg/L)	PO_4^{3-} (mg/L)	NH_4^+ (mg/L)	NO_2^- (mg/L)	NO_3^- (mg/L)	T-N (mg/L)
Stages						
Influent	210.0	2.8	23.1	0.0	0.0	44.3
Anaerobic 1	24.4	23.3	20.0	0.0	0.0	
Anaerobic 2	13.6	24.5	19.9	0.0	0.0	30.9
Oxic 1	17.7	7.7	11.5	1.4	3.4	
Oxic 2	16.6	0.5	2.3	0.3	15.1	
Oxic 3	16.1	0.2	2.3	0.0	16.4	
Oxic 4	15.9	0.0	2.9	0.0	15.5	21.3
Anoxic 1	18.8	0.0	1.1	1.3	7.3	15.3
Anoxic 2	21.6	0.0	0.0	1.3	5.4	14.7
Oxiic 5	18.4	0.0	0.0	1.5	7.5	
Effluent	16.1	0.0	0.0	1.5	7.5	13.3

* SRT=10 days, sludge recycling ratio=0.5, HRT=10 hrs

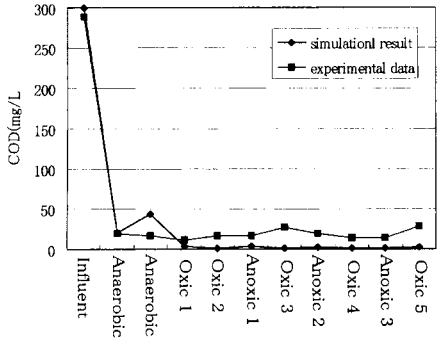

Figure 3. Experimental and simulated COD_s concentration in each stage in AOAO process ($Q_1 : Q_2 = 0.8 : 0.2$)

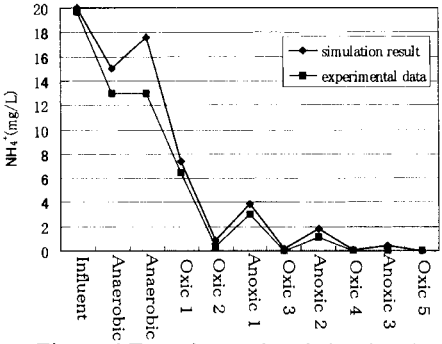

Figure 4 Experimental and simulated NH_4^+ concentration in each stage in AOAO process ($Q_1 : Q_2 = 0.8 : 0.2$)

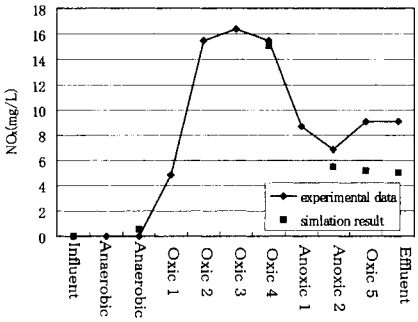

Figure 5. Experimental and simulated NO_x concentration in each stage in AOAO process ($Q_1 : Q_2 = 0.8 : 0.2$)

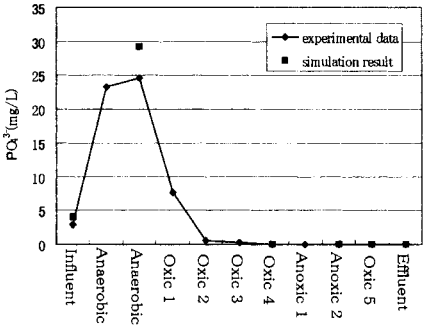

Figure 6. Experimental and simulated PO_4^{3-} concentration in each stage in AOAO process ($Q_1 : Q_2 = 0.8 : 0.2$)

3.2 Multiple stages EBNR process

The operational condition used in run1 gave the least T-N concentration in the effluent. The nutrients concentration in each stage under a steady state condition was summarized in Table 5. The removal efficiency for COD, T-P and T-N were 96.6, 100 and 76.4%, respectively. Figures 7-10 compare the profiles of the nutrient concentration from the experimental results and model simulation. Because the wastewater was fed into anoxic tanks by a step-feed mode, the nitrate in the effluent could be cut down until 7.8 mg/L. Furthermore, the alkalinity would be fed back due to denitrification in anoxic zones. The pH value in the effluent reached 7.5 without alkalinity control and external chemicals addition.

Table 5
Steady-state experimental results in multiple stages EBNR process under $Q_1 : Q_2 : Q_3 = 0.7 : 0.2 : 0.1$

Nutrients	COD_s (mg/L)	PO_4^{3-} (mg/L)	NH_4^+ (mg/L)	NO_2^- (mg/L)	NO_3^- (mg/L)	T-N (mg/L)
Stages						
Influent	288.0_{total}	3.6	19.7	0.0	0.0	42.1
Anaerobic 1	18.6	20.3	13.0	0.0	0.0	
Anaerobic 2	17.1	23.9	12.9	0.0	0.0	31.7
Oxic 1	11.7	12.2	6.4	0.6	5.4	
Oxic 2	16.7	6.8	0.3	0.9	10.3	25.3
Anoxic 1	16.8	4.9	3.0	0.8	5.6	
Oxic 3	26.8	2.5	0.0	0.4	8.4	15.6
Anoxic 2	19.1	1.4	1.1	0.4	6.7	
Oxic 4	13.7	0.6	0.0	0.1	7.6	11.3
Anoxic 3	14.2	0.5	0.0	0.1	7.9	
Oxic 5	27.8	0.3	0.0	0.0	7.8	
Effluent	9.8	0.0	0.0	0.0	7.8	9.9

* SRT=10 days, sludge recycling ratio=0.5, HRT=9.5 hrs

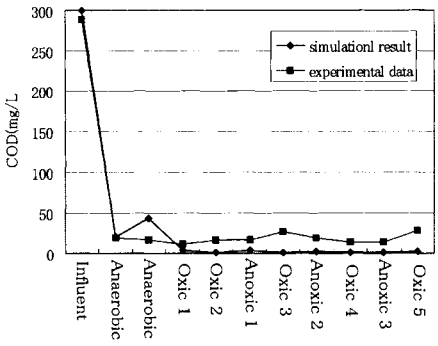

Figure 7. Experimental and simulated COD concentration in each stage in multiple stages EBNR process ($Q_1 : Q_2 : Q_3 = 0.7 : 0.2 : 0.1$)

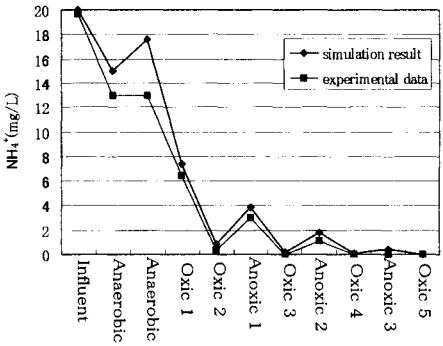

Figure 8. Experimental and simulated NH_4^+ concentration in each stage in multiple stages EBNR process($Q_1 : Q_2 : Q_3 = 0.7 : 0.2 : 0.1$)

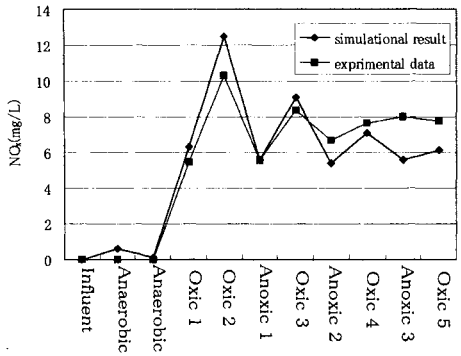

Figure 9. Experimental and simulated NO_x concentration in each stage in multiple stages EBNR process ($Q_1 : Q_2 : Q_3 = 0.7 : 0.2 : 0.1$)

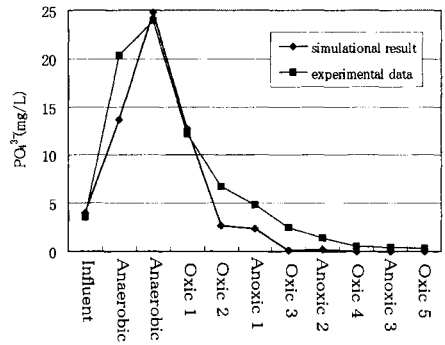

Figure 10. Experimental and simulated PO_4^{3-} concentration in each stage in multiple stages EBNR process ($Q_1 : Q_2 : Q_3 = 0.7 : 0.2 : 0.1$)

The T-N removal efficiency in run1 was higher than that in run2 or run3. Because of the low soluble COD remained in the anaerobic and anoxic effluent (Figure 7), ammonium nitrogen could be almost completely removed in the following aerobic tank. With the external addition of a carbon source, a higher specific denitrification rate and lower NO_x (NO_2^-+NO_3^-) concentration were achieved.

3.3 Comparison of process performance between AOAO process and multiple stages EBNR process

Under the same operational conditions, the optimal nutrient removal efficiency between the AOAO process and the multiple stages EBNR process was compared and shown in Table 6.

Table 6
Comparison of the optimal nutrient removal efficiency and HRT between AOAO and multiple stages EBNR process

Initial nutrients concentration of wastewater	Process and optimal step feed ratio	HRT	COD		T-N		T-P	
			residual con. (mg/L)	removal eff. (%)	residual con. (mg/L)	removal eff. (%)	residual con. (mg/L)	removal eff. (%)
COD_{total} (300 mg/L) T-N (40 mg/L) T-P (5 mg/L)	AOAO $Q_1 : Q_2$=0.8 : 0.2	10	23.1	92.3	12.0	70.0	0	100
	Multiple Stages EBNR process $Q_1 : Q_2 : Q_3$ = 0.7 : 0.2 : 0.1	9.5	10.2	96.6	9.4	76.4	0	100

* SRT=10 days, sludge recycling ratio=0.5

According to Table 6, the COD and T-N removal efficiency in multiple stages EBNR process at an optimal step feed ratio of 0.7 : 0.2 : 0.1 was slightly higher than those in AOAO process at an optimal step feed ratio of 0.8 : 0.2. It proved that multiple stages EBNR process could enhance the COD and T-N removal efficiency without T-P removal efficiency decline. Further higher T-N removal efficiency could also be obtained by adding external pure carbon source (Q_4) based on proper C/N ratio in the last anoxic tank.

3.4 Mathematical model simulation

The simulated results were shown in Figures 3-10. Figures 3 and 7 show the variation of the residual soluble COD in AOAO and multiple stages EBNR process, respectively. Soluble COD was the sum of the fermentable, readily biodegradable organic substrate (S_F) and fermentation products (S_A). According to the calculations, the experimental data and simulation results were almost the same. The variations of sum of the ammonium and ammonia concentration (S_{NH4}) in these two EBNR processes were shown as Figures 4 and 8. The S_{NH4} concentration would increase due to the hydrolysis of organic nitrogen in the anaerobic effluent and would decrease in the latter reactors. But the experimental data in anaerobic tank were higher than the simulation values in Figure 4. This might be probably caused by the fast hydrolysis rate of the soluble or particulate organic nitrogen. According to Figures 5 and 9, the nitrite and nitrate nitrogen simulated concentration (S_{NO3}) would increase in the anaerobic influent due to the recycling of the sludge from final clarifier, and it would decrease in the anaerobic effluent due to the denitrification in anaerobic tank. Then S_{NO3} would reach the highest concentration in the aerobic effluent due to nitrification. Originally, it was assumed that heterotrophic microorganisms under anaerobic conditions were capable of fermentation in ASM2d. Although these two processes might possibly cause growth of

heterotrophic microorganisms, they were introduced as a simple transformation process, and the growth was ignored. But the heterotrophic microorganisms growth was introduced in model here. The reduction factor for denitrification was assumed to be equal to 0.04 in anaerobic reactor to proceed with the simulation. Moreover, the phosphate concentration (S_{PO4}) would increase in the anaerobic effluent due to releasing of phosphate and decrease in the latter anoxic and aerobic effluent, as shown in Figures 6 and 10. A good fitness between experimental and simulated results was shown in these two figures. And according to the good fitting results, it was indirectly proved that there existing the denitrifying PAO in the system.

The components that were not analyzed empirically could be also calculated from model simulation. Figures 11 and 12 show that the heterotrophic microorganisms were the predominant species in microbial community. Furthermore, there was no significant difference in the amount of the suspended biomass between AOAO and multiple stages EBNR process. This finding might be caused by the same SRT, the similar reaction tank arrangement and the similar organic loading.

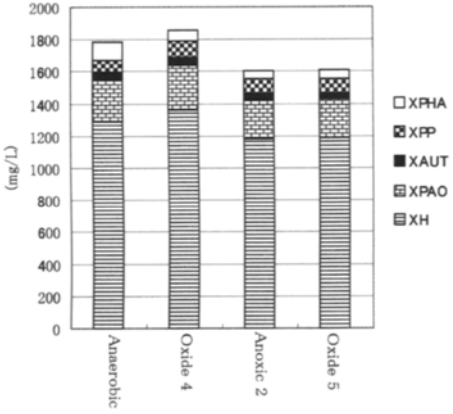

Figure 11. Suspend biomass, X_{PP} and the X_{PHA} concentration in AOAO process ($Q_1 : Q_2 = 0.8 : 0.2$)

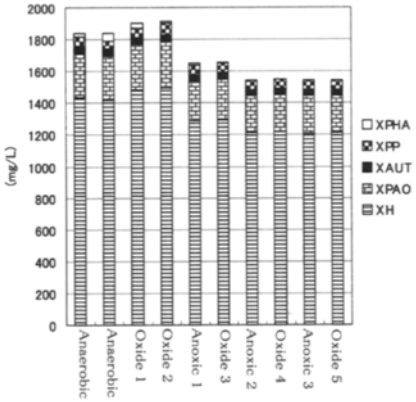

Figure 12. Suspended biomass, X_{PP} and the X_{PHA} concentration in multiple stages EBNR process ($Q_1 : Q_2 : Q_3 = 0.7 : 0.2 : 0.1$)

4. CONCLUTION

The N/P removal efficiency in an EBNR process was optimized by compartment reconfiguration and the stepwise feeding strategy. In comparison to AOAO process with a step feed ratio of 0.8 : 0.2, a better effluent water quality was obtained in multiple stages EBNR process with a step feed ratio of 0.7 : 0.2 : 0.1. The effluent nutrients concentration of T-N and T-P meets the effluent regulations of European and Japan without alkalinity control and recirculation of nitrified supernatant liquid between each individual zone in multiple stages EBNR process. A mathematical model based on ASM2d was successfully adopted to simulate these two EBNR processes. A good correlation between the experiment data and simulated values was obtained. Analysis on the microbial populations for nitrogen and phosphorus removal could also be calculated from model simulation.

REFERENCES

1. ASCE & WEF, Design of Municipal Wastewater Treatment plants, ASCE Manual and Report on Engineering, 1992.
2. J. L. Su and C.F. Ouyang, Proceedings of IAWQ 5th Asian Regional Conf., (1995) 581.
3. D. Orhon and N. Artan, Modeling of Activated Sludge System, USA, Pennsylvania, 1994.
4. M. Henze, W. Gujer, T. Mino, T. Matsuo, M.C. Wentzel, G.v.R. Marais and M.C.M.V. Loosdrecht, Wat. Sci. & Tech., 39(1) (1999) 165.
5. H. Zhao, O.J. Hao, and T.J. McAvoy, Wat. Sci. & Tech., 39(1) (1999) 227.
6. APHA, AWWA and WPCF, Standard Methods for the Examination of Water and Wastewater, 16th, Washington, D.C., 1985.
7. M. Henze, C.P.L. Grady Jr, W. Gujer, G.v.R. Marais, T. Matsuo, Activated Sludge Model No.1, IAWPRC task group on mathematical modeling for design and operation of biological wastewater water treatment, 1986.
8. K. Fujie, H.Y. Hu, B.R. Lim and H. Xia, Wat. Sci. & Tech., 35(7) (1997) 79.

Upgrading of waste stabilization pond to baffled reactor for domestic wastewater treatment

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Waste Stabilization Pond (WSP) is one of the treatment systems that widely used to serve urban population ranging from 5,000 to 450,000 in Malaysia. However most of the WSP systems do not function well due to overloading, improper and poor design or poor maintenance. The effects of poorly designed or inadequate maintenance, however, can be quite significant, in addition to failing to protect the receiving water, such as odor release from both anaerobic and facultative ponds, fly breeding in pond systems and floating macrophytes or emergent vegetation in facultative and maturation ponds leading to mosquito breeding, in extreme cases the ponds can silt up and completely disappear. In viewing this scenario and its associated problems, there are at least two main strategies, which can be applied in order to achieve the effluent quality required by the authority, i.e. rehabilitation and upgrading scheme of WSP. In this study baffled reactor system was used as an upgrading scheme for WSP. The objective of this study was to compare the performance of three types of baffled reactors in terms of COD removal and mixing patterns. Three laboratory-scaled baffled reactors were constructed with liquid volume of 90, 40 and 90 liters, respectively. Different type of operating conditions was applied to each reactor, which was aerobic for Reactor A, anaerobic for Reactor B and anoxic for Reactor C. COD was monitored with variation in HRT from 1 to 5 days on plug-flow system. Tracer study was also conducted to observe the flow regime using lithium chloride. In Reactor A, 89% of COD was removed at HRT of 2.5 days, and 75% COD removed for 1 day HRT. In Reactor B, 50% of COD was removed at 2.5 days HRT and 25% of 1 day HRT. In Reactor C, 91% COD was removed at HRT of 2 days and 76% at HRT of 1 day. The study has experimentally demonstrated that baffled reactors could be effectively used to upgrade WSP in order to achieve higher effluent standards, particularly using Reactor A.

1. INTRODUCTION

At present, Malaysia has almost 8,000 small-sized sewage treatment plants, and approximately 7,500 km length of sewer line. This sewerage system is mainly concentrated on urban areas covering for approximately 12 million populations (total population of Malaysia is slightly more than 22 million). In terms of types of technology used for treatment

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processes, it can be concluded that the standard is relatively conventional and primarily using pond systems, as illustrated in Table 1. Of the 4,539 public wastewater treatment plants maintained by a national sewerage company, i.e. Indah Water Konsortium (IWK), 55% are communal septic tanks, 15% are Imhoff tanks, 10% are pond systems and 20% are mechanical systems [1].

According to the *Sewerage Services Report 1994-97* [2], 2,204 or 48.6% plants out of the 4,539 plants taken over by IWK as of December 1997 were serving populations of less than 150 each, while 1,706 or 37.6% were serving populations ranging from 150 to 2,000. These figures indicated that 86.2% of the plants, mainly communal and septic tanks and Imhoff tanks were serving not more than 2,000 populations each.

One of the treatment systems that are presently used to serve urban population ranging from 5,000 to 450,000 is Waste Stabilization Ponds (WSP). WSPs are shallow man-made basins into which wastewater flows and from which, after retention time of many days (rather than several hours in conventional non-pond-based treatment processes), a well-treated effluent is discharged. WSP systems comprise a series of ponds: (a) anaerobic, (b) facultative and (c) several maturation ponds. The latest developments in research on WSP show the interest to include anoxic and algal ponds, as well as reservoir mainly for agricultural irrigation purposes [3-5]. The advantages of WSP systems particularly for hot climates and developing countries, can be summarized for its:- (a) simplicity (b) low cost (c) high efficiency with proper design and maintenance (d) robust (e) relatively reliable.

However most of the WSP systems in Malaysia do not function well. This may simply be due to overloading, but it can often be the result of the various factors associated to improper physical design, poor design or poor maintenance. The effects of poorly designed or inadequate maintenance, however, can be quite significant, in addition to failing to protect the receiving water, such as: (a) odor release from both anaerobic and facultative ponds (b) fly breeding in pond systems (c) floating macrophytes or emergent vegetation in facultative and maturation ponds leading to mosquito breeding, in extreme cases the ponds can silt up and completely disappear.

In viewing this scenario and its associated problems, there are at least two main strategies, which can be applied in order to achieve the effluent quality required by the authority, i.e. (a) rehabilitation (b) upgrading scheme for WSP. Upgrading of the existing WSP for municipal wastewater treatment in Malaysia can be viewed from multiple dimensions and reasons. In this study, the emphasis will be given to integrated pollution control in the upgrading scheme, in which all aspects such as effluent quality standard, sludge disposal, level of technology, ease of upgrading, odor control, land availability, maintenance, cost-effective and other non-financial factors have been considered.

Table 1
Wastewater treatment facilities in Malaysia [1]

Type of technology	%
Commercial septic tank	55
Imhoff tank	15
Oxidation tank/aerated lagoons	10
Mechanical treatment	20

In the future, the Malaysian Government may impose new and more stringent effluent standards for wastewater treatment facilities, in order to protect public health and the natural aquatic environment. At present almost all WSP facilities have been designed based on the Environmental Quality (Sewage & Industrial Effluents) Regulations 1979 for Standard A (BOD 20 mg/l, SS 50 mg/l), or Standard B (BOD 50 mg/l, SS 100 mg/l). The standards are mainly used to regulate the disposal of effluent to receiving waters. Based on the trend and development in most developed countries, the Government may introduce nutrient removal standards in the near future. To date the design objective for most of the WSP have been primarily focused on organic and SS removal. Thus, the upgrading scheme of the WSP in the future should include nutrient removal, or specific pollutants in order to meet the requirements set by Government.

2. BAFFLED REACTOR FOR UPGRADING WSP

Baffled reactor, aerobic, anaerobic or anoxic system, offers an economical solution to the treatment of domestic wastewater. Its simplicity in construction and has less moving mechanisms will be suitable for upgrading of many small-sized WSP in Malaysia. The baffled uses a series of vertical baffles to force wastewater to flow under and over them as it passes from the influent to the effluent. The microorganims within the reactor tend to rise and settle but move horizontally at a relatively slow rate. Therefore the wastewater will come into contact with a large active biomass as it passes through the baffles.

The baffles can also serve to develop biofilm thus the reactors achieve a high reaction rate per unit reactor volume in terms of kgCOD/m^3d by retaining the biomass, or SRT, in the reactor independently of the incoming wastewater, or HRT. This is in contrast to continually stirred tank reactors, thus reducing reactor volume and ultimately allowing the application of high volumetric loading rates, e.g. 10-40 kgCOD/m^3d [6].

In relation to WSP upgrading scheme, baffled reactors have been identified as one of the treatment processes to be employed in Malaysia, thus it requires further investigation into its scientific details, such as design parameters, system dynamic and operational details. UTM is actively involved in this study to provide the scientific support within three stages: (a) to evaluate the most cost-effective type of baffled reactors to be employed:- aerobic, anaerobic or anoxic, (b) to optimize the treatment processes using various advanced techniques, such as computational fluid dynamic (CFD), molecular microbial ecology and membrane bioreactor, (c) to establish scientific and design input to the local industry in wastewater sector.

The main objective of this paper is to present the main portion of first stage of the study, i.e. to comparatively demonstrate the performance of the three baffled reactors for COD removal. Tracer studies were also carried out to determine the mixing patterns.

3. MATERIALS AND METHODS

Three lab-scale baffled reactors made of perspex were constructed, namely A, B and C with a liquid volume of 90, 40 and 90 liter respectively. Reactor A was designed for aerobic system, B for anaerobic and C for hybrid aerobic-anaerobic systems and operated at room temperature, on average at 29°C. The room temperature was required in this study to reflect the simplicity of baffled reactors system in order to have a cost-effective dimension.

In Reactor A as shown in Fig. 1, an air pump was used to transfer the air from surroundings to the bulk wastewater at the rate of 1000 ml/min. Perforated teflon tubes were placed in each compartment to distribute the air. Reactor B, as schematically shown in Fig. 2, was anaerobically operated, divided into four compartments, which were, separated by standing baffles. Gas outlet was design for each compartment, and collected using displacement method. Reactor C as shown in Fig. 3 was operated under facultative condition with two compartments, which consists of 6 hanging baffles in the aerobic compartment. A gas outlet was provided for anaerobic compartment.

Wastewater samples were taken from a WSP in UTM campus, which collected wastewater from mainly cafeteria and student accommodations. The original concentration of COD was relatively low in the range of 80 to 100 mg/l. In order to increase the concentration to a desired level, synthetic wastewater was added which comprised a mixture of glucose and glutamic acid with a weight ratio of 1:1.

The ratio was used because results from laboratory experiment showed that the COD value was quite consistent from the first day until the third day of experiment as shown in Table 2. It was also verified that every 0.1 g/l solution of glucose or glutamic acid gave 100 mg/l COD. Based on these observations, the desired COD concentrations were varied by weighing a calculated amount of powdered glucose/glutamic acid and diluting it in distilled water.

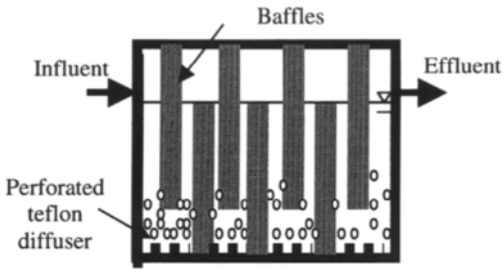

Figure 1. Reactor A – Aerobic baffled

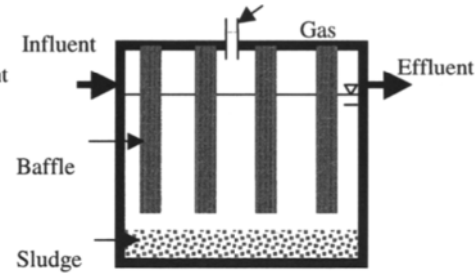

Figure 2. Reactor B – Anaerobic baffled

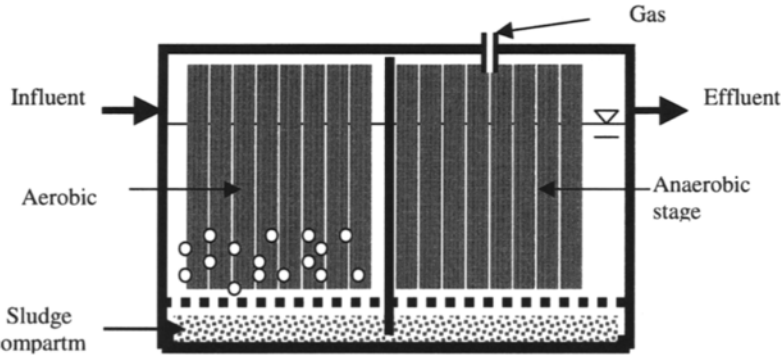

Figure 3. Reactor C – Aerobic-anaerobic baffled

The feed influent was placed in a refrigerator at 4°C. Stock buffer solution as shown in Table 3 was used to maintain pH of the reactors between 6.6 to 7.2. Trace elements were also added to the feed solution in order to provide sufficient nutrients for the microorganisms as shown in Table 4.

COD was measured for the influent and effluent, on daily basis. TSS and VSS were measured weekly. All parameters were measured using the *Standard Methods for Examination of Water and Wastewater* [8].

Tracer studies were carried out to determine the flow pattern within the reactors and the degree of mixing. Lithium chloride was used as a tracer solution, which was injected into the influent line using a 10 ml syringe. The concentration of LiCl was 5 mg/l. The samples were collected for a period of 4 times with respect to its theoretical HRT, and then analyzed for Li concentration using a flame photometer. Tracer studies were conducted for each reactor with and without biomass at a different HRT, as described in Tables 5 and 6.

Table 2
Stability of glucose and glutamic acid

Weight;Glutamic:Glucose		1:1	1:2.5	1:5	1:7.5
Day	1	165	165	165	165
	2	163	156	155	153
	3	160	142	149	149

Table 3
Composition of stock buffer solution (pH=6.8)

Compounds	Unit	Concentration
Na_2HPO_4	g/l	0.33
NaH_2PO_4	g/l	0.37
K_2HPO_4	g/l	0.40
KH_2PO_4	g/l	0.40
NH_4PO_4	g/l	0.013
Tracer element solutions*	ml/l	0.1

Table 4
Composition of Trace Element Solution [7]

Substances	Concentration (g/l)
$MgSO_4.7H_2O$	5.0
$FeCl_3.4H_2O$	15.0
$CaCl_2.6H_2O$	15.0
KCl	5.0
$CoCl_3.6H_2O$	5.0
Na_2MOO_4	0.2
$CuSO_4$	0.2
$ZnSO_4$	0.2
$NiSO_4$	0.2

Table 5
Effects of HRT on mixing patterns (without biomass)

	HRT (hr)	Active Vol (m^3)	Dead Vol (m^3)	Dead Zone (%)
Reactor A	17.2	0.93	0.07	6.6
	34.5	0.94	0.06	5.8
	59.6	0.97	0.03	3.3
Reactor B	17.1	0.92	0.08	7.8
	34.3	0.95	0.05	4.9
	64.6	0.98	0.02	2.2
Reactor C	21.2	0.84	0.16	15.8
	40.0	0.84	0.16	15.8
	64.6	0.85	0.15	14.9

Table 6
Effects of HRT on mixing patterns (with biomass) and COD removal

	HRT (hr)	Active Vol (m^3)	Dead Vol (m^3)	Dead Zone (%)	COD Removal (%)
Reactor A	21.8	0.92	0.08	7.8	70
	35.6	0.95	0.05	4.9	76
	60.1	0.98	0.02	1.9	89
Reactor B	22.2	0.95	0.05	5.3	25
	34.8	0.95	0.05	4.6	35
	52.9	0.96	0.04	3.5	50
Reactor C	20.0	0.84	0.16	15.6	72
	41.5	0.85	0.15	15.2	80
	72.7	0.86	0.14	14.4	90

5. RESULTS AND DISCUSSION

Tables 5 and 6 show the results of tracer studies using LiCl. For tracer study without biomass as shown in Table 5, on average the dead zone and the dead volume were higher in the Reactor C. On the other hand Reactor A has shown the lowest dead zone which indicates that mixing or contact time between influent and biofilm, and other biological transformations will be at the longest period. As shown in Table 6, the mixing and contact time in the Reactor A will proportionally contribute to the COD removal: 70%, 76% and 89% for HRT of 21.8, 35.6 and 60.1 days, respectively.

The mixing patterns were very significant for WSP upgrading since many studies have shown that the performance of organic removal in WSP has been degrading due to hydraulic short-circuiting and accumulation of sludge at various points in the ponds thus increased the dead zone [6]. The finding of present study, however, needs to be further verified preferably in three-dimensional graphical presentation using more advanced techniques, such as CFD. In short Tables 5 and 6 illustrated the importance of long HRT values to the degree of treatment in baffled reactors, in which it can be concluded that the longer the HRT the higher the degree of COD removal, particularly in Reactors A and C.

Since the scope of the present study was limited to hydrodynamic and its correlation to COD removal, thus no further investigation was conducted to study the microbial community dynamic in the reactors. However, the reduction of COD can be used to postulate that the microbial transformations occurred due to complex microbial community, especially the mutualistic relationship between pond algae and pond heterotrophic bacteria [9].

At constant COD value of 500 mg/l, all baffled reactors demonstrated the positive correlation between HRT and COD removal as shown in Tables 7, 8 and 9. It shows that the reduction in HRT values was negatively proportional to the COD removed, as also demonstrated in the Tables 5 and 6. It can be concluded that as the percentage of dead zones decreased the percentage of COD removal increased. This situation implied that mixing patterns influenced the reactor performance. Reactors A and C (partly) were operated aerobically so the diffused air contributed to the completely mixed process. As Reactor A has the lowest percentage of dead zones compared to Reactor C, thus it can suggested that the design and baffles arrangement of Reactor A was more effective in reducing the volume dead zone, as well as in increasing the microbial transformation for COD removal. This result implied that the percentage of COD removal could be increased by increasing the HRT since dead zones will be minimized.

Table 7
Variation of HRT at 500mg/l COD influent for Reactor A

HRT (days)	COD effluent (mg/l)	Loading (kg COD/m^3.day)	% COD removed
5.00	90	0.100	82
4.50	82	0.111	84
4.00	95	0.125	81
3.50	103	0.143	79
3.00	114	0.167	77
2.50	55	0.200	89
2.00	98	0.250	80
1.50	110	0.333	78

Table 8
Variation of HRT at 500mg/l COD influent for Reactor B

HRT (days)	COD effluent (mg/l)	Loading (kg COD/m^3.day)	% COD removed
5.00	300	0.100	40
4.50	330	0.111	34
4.00	290	0.125	42
3.50	350	0.143	30
3.00	390	0.167	22
2.50	250	0.200	50
2.00	300	0.250	40
1.50	325	0.333	35
1.00	375	0.500	25

Table 9
Variation of HRT at 500mg/l COD influent for Reactor C

HRT (days)	COD effluent (mg/l)	Loading (kg COD/m^3.day)	% COD removed
5.00	85	0.100	83
4.50	100	0.111	80
4.00	95	0.125	81
3.50	110	0.143	78
3.00	50	0.167	90
2.50	120	0.200	76
2.00	45	0.250	91
1.50	100	0.333	80
1.00	120	0.500	76

Figures 4 and 5 showed that Reactor C was capable of removing the COD as high as 91 percent whilst Reactor C has the lowest performance. Reactor A and C (partly) which were operated under aerobic conditions were considered consistent in removing COD for various levels of organic loading. Reactor B, which was operated under anaerobic condition at room temperature of about 29°C, was only capable of removing COD at a maximum of 50 percent.

Overall performance of Reactor B was rather unsatisfactory. This was due to system design employed in this study in which many environmental factors were not controlled and allowed to follow ambient conditions. Temperature, for example, at 35°C (compared to the average of 29°C for the present study) was the most suitable for anaerobic process for municipal wastewater treatment[10]. The organic loading of 0.5 kgCOD/m^3.day, which was significantly low, used in this study also contributed to the unsatisfactory COD removal. However anaerobic baffled reactor was suitably employed for high-strength waste. Anaerobic baffled reactor has several advantages over well-established systems such as the upflow anaerobic sludge blanket and anaerobic filter, includes better resilience to hydraulic and organic shock loadings, longer biomass retention times, lower sludge yield and the ability to partially separate between the various phases of anaerobic catabolism [11].

6. CONCLUSION

The present study has been focussed on comparing the performance of three baffled reactors to be used for WSP upgrading scheme in Malaysia. Reactors A and C showed the highest COD removal occurred at HRT of 2.5 and 3 days, respectively with organic loading range from 0.1 to 0.5 kgCOD/m^3.day. Reactor B has achieved the lowest COD removal mainly due to low temperature and low organic loading. On hydrodynamic part, the study has shown that the arrangement of baffles in Reactor A was the most effective in reducing the dead zone. The aerobic process through air diffusion improved mixing, and improved microbial transformations thus resulted in relatively better COD removal. Therefore it can be concluded that for the treatment of domestic wastewater which normally has a COD concentration in the range of 100 to 500 mg/l in tropical countries, the aerobic baffled reactors arranged as similar to Reactor A is more appropriate and recommended.

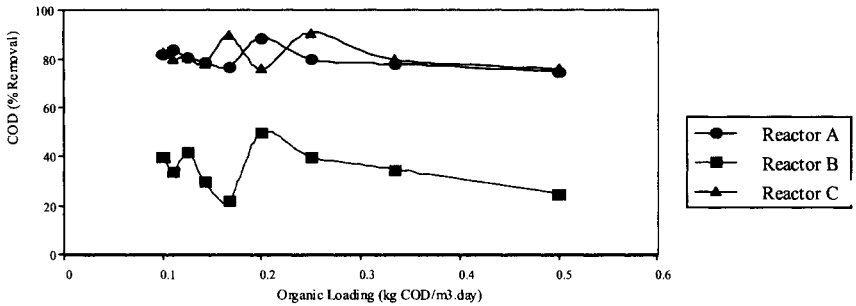

Figure 4. Graph COD removal vs organic loading

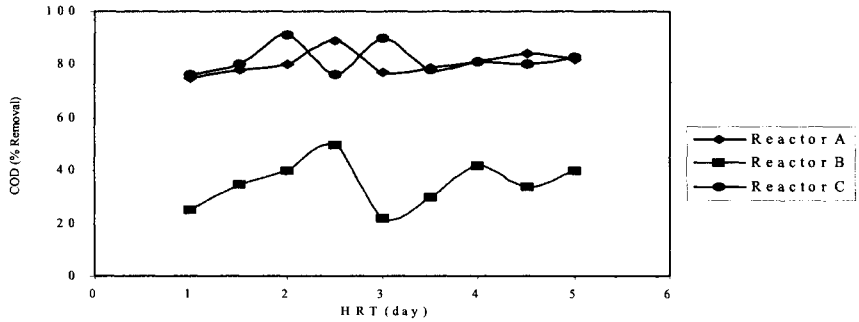

Figure 5. Graph COD removal vs HRT

REFERENCES

1. M.H.A. Hamid and M.Z. Muda, Proc. Nat. Symp. Advanced Wastewater Treatment, UTM, Kuala Lumpur (1999).
2. Malaysian Ministry of Housing & Local Government, Sewerage Services Report 1994-97, Kuala Lumpur, 1998
3. Almasi and M.B. Pescod. Wat. Sci. Tech. 33 (1996) 125.
4. Y. Nurdogan and W.J. Oswald. Wat. Sci. Tech., 33 (1996) 229.
5. D.D. Mara, H.W. Pearson, S.A. Silva, R. De Oliveira, A.L.C. Araujo, R.E. De Olievera and J. Soares. Wat. Sci. Tech., 33 (1996) 243.
6. H.E. Salter, C.T. Ta, S.K. Ouki and S.C. Williams. Proc. IAWQ Specialized Conf. on WSP, Morocco (1999).
7. A.D. Eaton, L.E. Clesceri and A.E. Greenberg (eds.), Standard Methods for Examination of Water and Wastewater, 19th Edition, APHA, AWWA and WEF, Washington, 1995.
8. M.A. Kassim. Effects of Media Types and Sizes on Performance of Anaerobic Fluidized Bed Reactors, Ph.D. Thesis, Univ. of Newcastle upon Tyne, England (1992).
9. D.D. Mara, G.P. Alabaster, H.W. Pearson and S.W. Mills (1992), Waste Stabilisation Ponds: A Design Manual for Eastern Africa, Lagoon Tech. Int., Leeds, 1992.
10. V.D. Berg and K.J. Keneddy (1983), Proc. 3rd. Int. Symp. on Anaerobic Digestion, Massachusetts (1983) 71.
11. W.P. Barber and D.C. Stuckey D.C. Wat. Res., 33 (1999) 1559.

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Potentials of vertical-flow constructed wetlands for septage treatment in tropical regions

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Constructed wetlands (CWs) have been proven to be an effective low-cost treatment system, which utilizes the interactions of emergent plants and microorganisms in the removal of pollutants. For wastewater treatment, CWs are typically designed to operate in either free-water surface or subsurface flow hydraulic patterns, while a vertical-flow operation is used to treat sludge or septage having high solid contents. This paper presents an assessment of the potentials of vertical-flow CWs for septage treatment in tropical regions where microbial reactions and plant growth rates are substantially high.

This study was conducted at the Environmental Research Station of AIT, using three pilot-scale CW beds, each with a dimension of 5 x 5 m^2. The CW beds consist of 65-cm sand-gravel substrata, supported by ventilated-drainage system and planting with narrow-leave cattails (*Typha augustifolia*). During the first year of operation, the CWs were operated at the solid loading rates (SLR) and application frequencies of 80 – 500 kg total solid (TS)/m^2.yr and 1 – 2 times weekly, respectively. The SLR of 250 kg TS/m^2.yr was found to result in the optimum treatment performances with respect to the TS, total chemical oxygen demand (TCOD) and total Kjeldahl nitrogen (TKN) removal efficiencies of 80, 96 and 92%, respectively, as well as less adverse effect on plant growth. At relatively high TS contents in the dewatered septage without any removal for a year, but permeability of the CW beds rarely decreased, probably due to the distribution of plant roots and rhizomes. It was also evident that there were high degrees of nitrification occurring in the CW beds in which the percolate had nitrate (NO_3^-) concentrations of 120 – 250 mg/L, while NO_3 contents in the raw septage were less than 10 mg/L. However, due to rapid flow-through of the percolates, there was little liquid retained in the CW beds, causing the cattail plants to wilt, especially during the dry season. In order for cattail plants to grow healthier, the operating conditions in the second year were rectified by ponding (or retaining) the percolate in the CW beds for periods of 2 and 6 days prior to discharge, but maintained the SLR of 250 kg TS/m^2.yr. This operating condition was obviously beneficial not only for mitigating plant wilting but also for increasing N removal through enhanced denitrification reactions in the CW beds, resulting in

the percolate NO_3 concentrations of 20 – 50 mg/L. Apart from the treatment performances, land requirement of the vertical-flow CWs was estimated to be only 32.5 m^2/1,000 capita with the low capital, operation and maintenance costs. The vertical-flow CW systems thus have a high potential for septage treatment in tropical regions where land areas are available.

LIST OF ABBREVIATIONS

BOD = Biochemical oxygen demand
CW = Constructed wetland
N = Nitrogen
NH_4 = Ammonium
NO_3 = Nitrate
SLR = Solid loading rate
TCOD = Total chemical oxygen demand
TKN = Total Kjeldahl nitrogen
TS = Total solids
TVS = Total volatile solid

1. INTRODUCTION

CWs are man-made systems aiming at simulating the treatment processes in natural wetlands by cultivating emergent plants e.g. reeds (*Phragmites*), bulrushes (*Scirpus*), and cattails (*Typha*) on sand, gravel, or soil media. Based on investigations of pilot and field-scale systems, CWs have been proven to be a promising treatment alternative characterized by low investment, operation and maintenance costs [1,2]. Therefore, utilization of CWs in waste treatment and recycling is currently of interest, including their ancillary benefits such as supporting primary production and enhancement of wildlife habitats. For several years, a number of CW systems have been employed to treat various kinds of wastewaters including, more recently, sludge from conventional treatment plants [3].

For sludge or septage dewatering, a vertical-flow mode of operation with a percolate-drainage system beneath CW beds is required. An advantage of CWs over conventional, unplanted sludge drying beds is the much lower frequency of dewatered sludge removal from the bed, allowing for several years of sludge accumulation prior to bed emptying. Furthermore, the percolating liquid is subjected to microbial reactions within the CW beds, enabling nitrification and higher removal efficiencies within the liquid. Septage treatment in CWs was conducted in laboratory-scale experimental units at Cemagref in Lyon, France [4], showing the promising treatment performance and ease of operations. Since 1996, the EAWAG and AIT have jointly undertaken research collaboration on "Septage Treatment by CWs and Attached-growth Waste Stabilisation Ponds" to test the feasibility of this treatment option and to establish design and operational guidelines. This article presents the 2-year experimental results of the CWs employed to dewater septage. Economic appraisal on the CW systems has also conducted to determine investment and operation costs suitable for developing countries.

2. MATERIALS AND METHODS

2.1 Experimental Setup

Configurations and Dimensions - Based on the site and literature surveys of the CWs treating sludge in Europe [3], it was evident that performances of these CW units were not dependent of length to width ratio, but more on distribution of sludge onto the bed surface. The AIT

pilot-scale CWS were then figured to be square in shape in order that septage could be uniformly distributed by a feeding system. The AIT CWs comprise of three vertical-flow units, each with a surface area of 5 x 5 m and lined with ferro-cement as shown in Figure 1.

Filter Media and Vegetation - Based on the suggestions of Cooper et al. [2], the filter media of CW units planting with reeds should have a depth of 80 cm with a 70-cm graded gravel layer and topped off with 10-cm coarse sand. Because length of the cattail roots is only about 30-40 cm, relatively shorter than reeds (50-60 cm), the substrata depth in these experiments was designed to be 65 cm. A 10-cm layer of fine sand, 15-cm layer of small gravel, and 40-cm layer of large gravel from top to bottom were used as substrata in each CW unit. A free board of 1 m was allowed for accumulation of the dewatered septage. On top of the sand layer, narrow-leave cattails (Typha augustifolia), collected from a nearby natural wetland, were planted in each CW unit at the initial density of 40-50 shoots/m^2. Cattails were selected because they are indigenous species and evidently growing better than reeds in most wetland areas of Thailand.

Ventilation and Percolation Systems - The bed support and drainage system consist of hollow concrete blocks, each with a dimension of 20 x 40 x 16 cm (width x length x hollow space), and perforated PVC pipes with a diameter of 20 cm at the bottom. Mounted on the drainage system are ventilation pipes of the same diameter and extending approximately 1 m over top edge of the units. Natural draught ventilation is required to avoid anaerobic conditions in the root zone and, hence, plant damage. The percolate of each CW unit was collected in a 3-m^3 concrete tank for sampling and analysis.

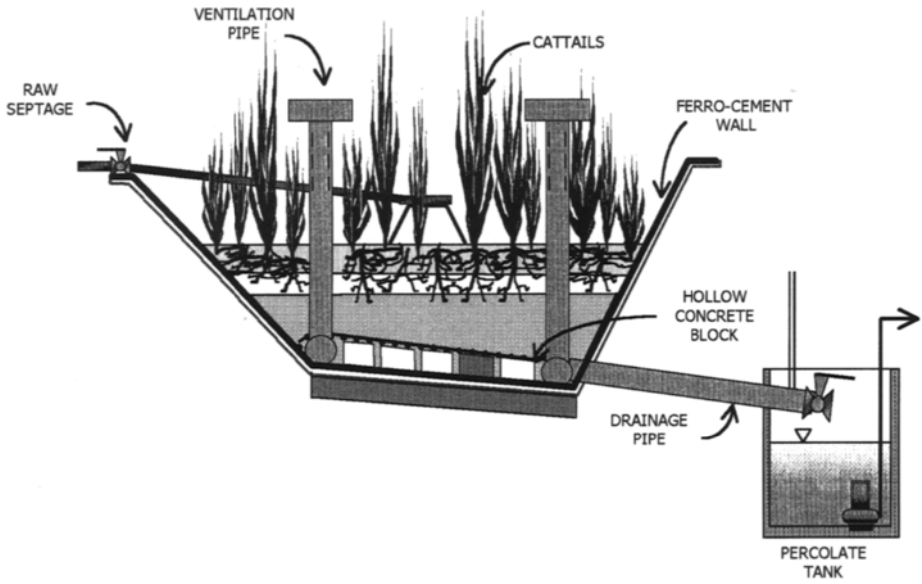

Figure 1. Schematic diagram of pilot-scale CW units

2.2 Operating Conditions

Cooper *et al.* [2] suggested solid loading rates (SLR) for reed beds treating excess sludge from a wastewater treatment plant in Europe to range from 30 – 80 kg total solids (TS)/m^2.yr and sludge loading frequency should be once a week. In tropical regions, it is expected that CWs could be loaded at the higher ranges of SLR.

Septage used in these experiments was transported from Bangkok city. To remove garbage and rags, the raw septage was passed through bar screen and then homogenized in two 4-m^3 mixing tanks before feeding to the CW units. The CW units were subjected to the operating conditions as shown in Table 1.

In Runs I – V, the CW percolates were allowed to flow freely into the percolate tanks soon after septage feeding. For Run VI, the percolate ponding conditions were maintained by closing the outlet valves according to the designed ponding periods. In stead of maintain the constant SLR, which would result in variations of volume loading, the septage was loaded at the constant volume of 8 m^3/week, corresponding to SLR range of 140 – 360 kg TS/m^2.yr in Run VI, which aims to ease the operational practices of CWs.

Samples collected from the CW units at different operating conditions were analyzed for the contents of TS, total volatile solids (TVS), suspended solids (SS), biochemical oxygen demand (BOD), TCOD, TKN, ammonium (NH_4), NO_3, and helminth eggs. Analytical methods for these parameters were according to Standard Methods for the Examination of Water and Wastewater [5].

3. RESULTS AND DISCUSSION

3.1 Characteristics of Bangkok Septage

During the 2-year observations, the Bangkok septage showed the characteristics as given in Table 2, based on septage samples during August 1997 – September 1999.

The Bangkok septage had solid and organic contents in the same ranges as those reported by U.S. EPA [6], but having relatively higher N contents. The low ratio of BOD to TCOD concentrations in the Bangkok septage showed the biodegradable fractions were mostly decomposed in the septic tanks. Because public hygiene and sanitation in Thailand, especially in the capital city, have significantly improved, the numbers of helminth eggs in the

Table 1
Operating conditions of pilot-scale CW units

Run	SLR (kgTS/m^2.yr)			Percolate	Frequency of	Periods of
	CW-1	CW-2	CW-3	ponding *	Septage application	Operation
I	250	125	80	No	twice-a-week	Apr. 97 – May 97
II	250	125	80	No	once-a week	May 97 – Jul. 97
III	250	125	80	No	twice-a-week	Aug. 97 – Dec. 97
IV	500	250	160	No	twice-a-week	Dec. 97 – Jan. 98
V	500	250	160	No	once-a week	Feb. 98 – Mar. 98
VI	250[a]	250[b]	250[c]	Yes	once-a week	Apr. 97 – Feb. 99
VII[+]	140 – 360	140 – 360	140 – 360	Yes	once-a week	Mar. 99 – Sep. 99

* Percolate was retained 10 –15 cm below dewatered septage layers in CW units

[+] To ease the operational practices, septage was loaded at the constant volume of 8 m^3/week, resulting in variations of SLR

[a] ponding period = 6 days, [b] ponding period = 2 days, [c] no ponding

septage samples were evidently lower than those observed in other developing countries. Therefore, the Bangkok septage could be classified as type "B" or low-strength fecal sludge as suggested by Strauss *et al.* [7].

3.2 First-year Results: Run I – IV Experiments

The results obtained from the first year operation during Run I – IV showed that removal efficiencies of TS, TCOD, TKN, and NH_4 to be mostly higher than 80%, while the average NO_3 concentrations increased from 8 to 180 – 260 mg/L (Table 3). Analyses of the CW performance are given below.

Solid Removal and Sludge Drying - Based on the results of Runs I – III, which had the same ranges of SLR, the frequencies of septage application did not have significantly effects on the TS removal efficiencies. The TS removal efficiencies achieved in the CW beds were similar to those of the sand drying beds treating the septage, which were experimented in parallel for comparisons [7,8]. At the end of the 4-month operation of Run II, the TS contents

Table 2
Characteristics of Bangkok septage samples

Parameter	Range	Average	Standard deviation
pH	6.7 – 8.0	7.5	0.6
TS (mg/L)	5,700 – 28,000	16,400	6,000
TVS (mg/L)	4,000 – 21,800	12,170	4,600
SS (mg/L)	3,150 – 21,600	13,600	5,800
BOD (mg/L)	600 – 5,500	2,800	1,400
TCOD (mg/L)	5,400 – 34,500	18,200	7,600
TKN (mg/L)	370 – 1,500	1,060	360
NH_4 (mg/L)	200– 590	390	110
NO_3 (mg/L)	8 – 20	12	3
Helminth eggs, no./g of sample	0 – 14	5	1

Table 3
Average TS, TCOD, TKN, NH_4, and NO_3 contents in CW percolate and removal [+]

Sample	Run	Unit No.	SLR kg TS/m^2.yr	Frequency No./week	Parameter [*], mg/L TS	TCOD	TKN	NH_4	NO_3
Raw septage[**]					16,300	16,000	830	340	8
Percolate	I	1	250	2	3,340 (81)	810 (97)	110 (95)	100 (90)	260
		2	125	2	3,610 (80)	570 (96)	62 (98)	44 (93)	190
		3	80	2	2,980 (83)	110 (98)	10 (99)	5 (98)	200
	II	1	250	1	2,640 (80)	300 (97)	62 (93)	46 (85)	180
		2	125	1	2,840 (80)	230 (98)	60 (96)	56 (80)	180
		3	80	1	3,640 (78)	210 (98)	45 (98)	32 (92)	210
	III	1	250	2	2,720 (88)	780 (95)	110 (94)	87 (88)	200
		2	125	2	2,700 (84)	460 (97)	100 (96)	36 (91)	190
		3	80	2	2,670 (86)	910 (94)	95 (99)	49 (79)	260
	IV	1	500	2	2,900 (81)	1,020 (93)	182 (87)	190 (69)	180
		2	250	2	3,600 (76)	800 (94)	140 (90)	100 (79)	220
		3	160	2	3,800 (80)	1,720 (88)	250 (79)	190 (52)	190

[+] Removal efficiencies as shown in parentheses depended on the characteristics of raw septage used in each experimental run.

[*] Average data were based on 12 composite samples taken from each experimental run.

[**] Raw septage data were averages of 72 samples of Run I to IV, during April 1997 – January 1998.

of the dewatered septage increased from 1 – 2% to 30 – 60% after about one week of dewatering. It was also observed that average heights of the dewatered septage layers were 1.5, 2.3 and 5.0 cm in the CW units operated at the SLRs of 80, 125 and 250 kgTS/m^2.yr, respectively.

From statistical analyses, the treatment efficiencies of Run III were higher than Runs II, and I probably because of the accumulated dewatered septage layers which contribute to better filterability and increased microbial reactions. It was also apparent from the results of Run IV that further increases in SLR resulted in decreased CW performance in term of TS removal. The high SLR corresponded to the large volume of applied septage, which were beyond the filtration capacity of the CW beds, hence the lower TS removal efficiencies. However, as a result of the filtering capacity, no helminth egg was found in the percolate samples of the CW units [7].

Organic Removal - During the first 8-month of Runs I – IV operations, the TCOD removal efficiencies ranged from 88 to 98%, resulting in the percolate TCOD concentrations of 210 – 1,720 mg/L (Table 3). Similar to the TS removal, the frequency of septage application did not have any significant effects on the TCOD removal efficiencies. It appeared that the TCOD removal depended on filtration capacity of the CW units rather than organic biodegradation at relatively short HRT. Another cause of the little biodegradation activity was the low biodegradability of the Bangkok septage, which had a BOD/COD ratio of 0.12.

N removal - Based on the results shown in Table 3, the TKN removal efficiencies of the CW units during Runs I – IV were mostly greater than 80% and having percolate TKN concentrations of 45 – 250 mg/L, while the NH_4 removal efficiencies were found in the range of 52 – 98%. The percolate NO_3 concentrations were increased significantly from 8 to 180 - 260 mg/L, probably because of the nitrification reactions. Moreover, the percolate DO concentrations of 2 – 4 mg/L supported the growth of the nitrifying bacteria. This result revealed the beneficial effects of the vertical-flow mode of operation and the ventilated-drainage system that enhanced the nitrification reactions.

Growth Patterns of Cattails - At the beginning of operation, the cattail plants in the CW units were 1.5-1.8 m in height. After two to three weeks of septage application, young roots and stems began to grow, but some cattail plants could not adapt to the septage, causing their leaves to turn yellow and died.

From observations, the cattail plants in the CW units showed a sign of water deficiency in Run I and were shocked during Run II, which was due to the once-a-week loading. From Run III, the plants could grow well because they became acclimated to the septage and septage loading was done twice a week.

After doubling the SLR to be 160, 250 and 500 kg/m^2.yr in Run IV, serious wilting symptoms and plant die-offs occurred in all CW units. The cattail plants in CW units no. 1 and 2 were manually harvested without removal of the dewatered septage layers, while no plant harvesting was implemented in unit no. 3.

3.3 Second-year Results: Run V – VII Experiments

At the end of Run IV, heights of the dewatered septage layers were increased to be 20 – 25 cm. The results obtained from the second year operation and beyond are shown in Table 4, and their analyses are given below.

Solid Removal and Mass Balance - After maintaining the doubled SLR and septage application at once-a-week in Run V, the TS removal efficiencies were in the same ranges of those obtained from Run IV, asserting that the TS removal of CWs is independent of the

septage application frequency. However, based on the results of Runs I – V, it was found that the SLR of 250 kg TS/m^2.yr resulted in the highest TS removal efficiencies. At the end of Run V (300 days of operation), the TS mass balances in each CW bed were analyzed as shown in Table 5. The accumulated TS inputs to CW units 1, 2, and 3 were 187, 115 and 112 kg TS/m^2, respectively. The average TS mass in dewatered septage amounted to 38 – 52% of the TS inputs, while about 11 – 12% of the TS inputs were in the percolate portion. The unaccounted TS of 36 – 50% were postulated to be due to biochemical reactions such as mineralisation, biodegradation, and TS accumulation in the CWs substrata.

The CWs operations in Run VI were maintained at the SLR of 250 kg TS/m^2.yr, while the CW percolates were withheld at the ponding periods of 6, 2 and 0 days in CW units 1, 2, and 3, respectively. It appeared from Table 4 that the percolate ponding periods did not have significant effects on the TS removal efficiencies, probably because the filtering capacity of the CWs did not increase via percolate ponding. In Run VII at the constant septage loading of 8 m^3/week corresponding to the SLR range of 140 – 360 kg TS/m^2.yr, it is apparent that no significant effect on the TS removal efficiencies in either CW units as compared to those resulted from Run VII. The CW units treating septage at this operating condition could, therefore, achieve the TS removal of 76 – 82%.

Organic Removal - The data of Run V confirmed that the SLR of 250 kg TS/m^2.yr yielded the highest TCOD removal of 96%. For Run VI and VII, the percolate ponding periods did not have any significant effects on the TCOD removal, similar to the TS removal efficiencies. It is postulated that sedimentation and filtration of organic particulate to be the

Table 4
Average TS, TCOD, TKN, NH_4, and NO_3 contents in CW percolate and removal [+]

Sample	Run	Unit No.	SLR kg TS/m^2.yr	Ponding days	Parameter [*], mg/L TS	TCOD	TKN	NH_4	NO_3
Raw septage[**]					18,500	16,000	1,000	440	6
Percolate	V	1	500	-	4,960 (82)	1,880 (94)	240 (82)	170 (52)	250
		2	250	-	6,030 (77)	850 (96)	120 (87)	110 (70)	320
		3	160	-	4,320 (77)	1,250 (91)	150 (83)	110 (68)	270
	VI	1	250	6	2,000 (86)	270 (98)	100 (89)	80 (81)	20
		2	250	2	2,400 (84)	400 (97)	150 (85)	100 (77)	53
		3	250	0	2,700 (81)	620 (96)	200 (80)	140 (69)	120
	VII[++]	1	140 – 360	6	2,600 (82)	320 (98)	106 (89)	80 (81)	22
		2	140 – 360	2	2,700 (78)	450 (97)	150 (84)	100 (70)	55
		3	140 – 360	0	3,300 (76)	780 (94)	220 (79)	140 (60)	130

[+] Removal efficiencies as shown in parentheses depended on the characteristics of raw septage used in each experimental run.
[++] Solid loading at the constant volume of 8 m^3/week
[*] Average data were based on 15 composite samples taken from each experimental run.
[**] Raw septage data were averages of 60 samples of Run V - VII, during February 1998 – September 1999

Table 5
TS mass balance in CW units after 300-day of operation

Balance	Unit no. 1 kg TS/m^2	%	Unit no. 2 kg TS/m^2	%	Unit no. 3 kg TS/m^2	%
Accumulated TS loading	187	-	115	-	112	-
Dewatered septage	93	50	60	52	43	38
Percolate	20	11	14	12	13	12
Unaccounted	74	39	41	36	56	50

major mechanisms responsible for TCOD removal in these CW units rather than biodegradation. The relatively low BOD/TCOD ratio of 0.1 – 0.2 in raw septage would likely be another cause of the scanty biodegradation.

N removal - The N removal efficiency of Run V at the SLR of 250 kg TS/m^2.yr was the highest. In Run VI, CW units 1 and 2, which had percolate, ponding had higher TKN and NH_4 removal efficiencies than CW unit 3 without percolate ponding. This phenomenon was probably due to the denitrification reactions occurring in the CW beds. CW unit 1, having the longest ponding period of 6 days, achieved the highest TKN and NH_4 removal efficiencies, while the percolate NO_3 concentration was the lowest. The N plant uptake by cattail plants could be another N removal mechanism in the CW units, as reported by Kootatep and Polprasert[10]. However, because the relatively high N loading of septage employed in these experiments, the N plant uptake rates of these CW beds were accounted for only 5 – 7% of the total N input[9]. Based on the results obtained from Run VII, it is evident that the variation of SLR subjecting to the N loading did not cause any adverse effect on TKN and NH_4 removal. It was found that the CW units could have the TKN and NH_4 removal efficiencies of 79 – 89% and 60 – 81%, respectively, while the percolate NO_3 concentrations were 22, 55 and 130 mg/L in CW no. 1, 2 and 3.

Effects on Cattail Growth and Percolate Flow - Due to inadequate water availability, severe wilting of the cattail plants were observed in Run V as a result of the doubled SLR and once-a-week septage application. The wilted cattails in CW units 1 and 2 were harvested prior to the start-up of Run VI. With percolate ponding in Run VI and VII, the cattails in CW units no. 1 and 2 grew much better than those in CW unit no. 3, which, because of no plant harvesting, had interferences from other weeds and dead cattails.

Even when the dewatered septage layers in the CW beds were 45 – 60 cm in height at the end of Run VII and the dewatered septage was not removed from the CW beds, there was no bed clogging as evidenced from the percolate flows during the course of operation. This phenomenon was presumably due to the continuous growth and distribution of the cattail roots and rhizomes in the dewatered septage layers and substrata, which helped to create porosity in the CW beds. It is obvious that the practice of septage dewatering in vertical-flow CWs without frequent removal of the dewatered septage should result in significant reduction of operating costs, and confirming its advantage over conventional sand drying beds.

Economic Appraisal - In accordance with the pilot-scale CW operating experiences gained to date, a preliminary economic appraisal was conducted to determine the capital and annual O&M costs for septage treatment. The preliminary economic appraisal revealed the land-free capital costs could be amounted to 5,300 US$ for each of CW unit, incorporating soil excavations, wood piling, ferro-cement lining, filter materials, vent pipes, cattail cultivation, piping and percolation system, and concrete percolate tank. The annual O&M costs for septage loading, harvesting of cattails (once or twice a year), and cleansing of units is estimated to 500 US$/unit, excluding of the costs of dried sludge removal which may require after 4 – 5 years of operation. At the optimum SLR of 250 kg TS/m^2.yr, each CW unit is able to treat septage generated from about 1,000 capita, having daily septage generation rate of 1 L/person and TS content of 18 – 20 g/L [6]. It could result in the capital and annual O&M costs of 5.3 and 0.5 US$/cap, respectively. In addition, the study suggested land requirement of the vertical-flow CW systems for septage treatment to be 32.5 m^2/1,000 persons.

4. CONCLUSIONS

Based on the 2-year experimental results, suitable strategies for the vertical-flow CW system treating septage were found to be: (i) SLR of 250 kg TS/m^2.yr or constant volume loading of 8 m^3/week; (ii) once-a-week septage application, and (iii) percolate ponding period of 6 days. These strategies resulted in optimum performance of CWs with respect to septage dewatering and contaminant removal from the percolating liquid, healthy and reliable plant growth, and ease of operations in which removal of the dewatered septage was not required. The percolate ponding significantly promoted the nitrification/denitrification reactions essential for N removal. Although more long-term data are required, the results generated to date indicated that the vertical-flow CW system is a promising technology for septage dewatering with low investment and operation costs.

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REFERENCES

1. Kadlec, R.H. and Knight, R.L. (1995). *Treatment Wetlands*, Lewis Publisher, New York.
2. Cooper, P.F., Job, G.D., Green, M.B., and Shutes, R.B.E. (1996). *Reed Beds and Constructed Wetlands for Wastewater Treatment*, WRc Swindon, Wiltshire.
3. Heinss, U. and Koottatep, T. (1998). *Use of Reed Beds for Faecal Sludge Dewatering.* EAWAG/AIT. Unpublished report.
4. Liénard, A. and Payrastre, F. (1996). *Pre Prints of the 5th International Conference on Wetland Systems for Water Pollution Control*, Vienna, 8 p.
5. APHA, AWWA, WPCF (1992). *Standard Methods for the Examination of Water and Wastewater*, 17th Edition, Washington, D. C.
6. United States Environmental Protection Agency (U.S. EPA) (1995). *Process Design Manual – Surfac e Disposal of Sewage Sludge and Domestic Septage*, Washington DC.
7. Strauss, M., Larmie, S.A., and Heinss, U. (1997) *Wat. Sci. & Tech.*, Vol. 35, No. 6, pp. 129-136.
8. Limsuwan, S. (1997). Operational criteria for septage dewatering in constructed wetlands, *Master Thesis*, EV-97-45, Asian Institute of Technology, Bangkok.
9. Pinsakul, A. (1997). Nitrogen removal in constructed wetland beds treating septage, *Master Thesis*, EV-97-2, Asian Institute of Technology, Bangkok.
10. Koottatep, T. and Polprasert, C., (1997). *Wat. Sci. & Tech.*, Vol. 36, No. 12, pp. 1-8.

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