Modeling the Behavior of Introduced Bacteria in the Activated Sludge Process

（活性汚泥プロセスに導入された細菌の挙動のモデル化に関する研究）

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General Introduction

The biological wastewater treatment began about a century ago, and is considered as an economical and well established technology. However, water quality requirements have become more stringent in recent years, as more pollutants are recognized, and as the public places more importance on water quality. Consequently, further improvements on wastewater treatment processes are required. The effective operation of biological wastewater treatment processes such as the activated sludge process mainly depends on bacterial populations and their activities involved in the processes. The bacterial populations have the ability to acclimate to fluctuations in the quality and/or quantity of influent, therefore, the improvement of biological wastewater treatment processes has been conventionally carried out by controlling operational factors, such as dissolved oxygen, sludge retention time, and load of organic compounds and nutrients, so as to proliferate and maintain desirable bacteria and their activities (indirect control of bacteria).

However, many wastewater treatment plants cannot respond quickly enough to produce the required standard of effluent and some problems as shown in Table 0.1 have never been completely solved by the conventional approaches although various wastewater treatment processes have been developed (Fujita and Ike, 1994; Fujita et al., 1996). Desirable bacteria for efficient treatment are usually present in relatively small numbers and their physiological characteristics cannot be manipulated by operational factors of the processes. For example, if little or no degrading bacteria are contained in the treatment processes, recalcitrant compounds will not be broken down. Even when degrading bacteria are present, they are not as effective as they could be because their growth rates are lower than those of the other bacteria present in the treatment environment. Bacteria without resistance are killed by the influx of toxicants.

In cases where well-acclimated bacterial consortia are not available, a current trend in wastewater treatment practices to solve the problems is the use of bioaugmented processes.

Table 0.1. Unsolved problems in wastewater treatment processes (Fujita and Ike, 1994)

1) Recalcitrant compounds (aromatics, haloaromatics, haloaliphatics, polycyclic aromatics, pesticides, etc.) cannot be degraded completely or efficiently.
2) Influx of toxic compounds (cyanide, heavy metals, antibiotic agents, etc.) lowers treatment efficiency.
3) Removal of nutrients (nitrogen and phosphorus) is not sufficient.
4) Treatment under extraordinary conditions (extremely low or high pH and/or temperature, high salinity, etc.) is impossible.
5) Constant control is difficult because of the fluctuation of environmental conditions (quantity and quality on influent, atmospheric temperature, etc.)
6) The problems of bulking caused by filamentous bacteria, and foaming caused by actinomycetes, are often observed in the activated sludge process.

Bioaugmentation, the introduction of desirable bacteria into a biological wastewater treatment process in which high concentrations of other bacteria are present, attempts to overcome the problems by significantly increasing the diversity and/or activity of desirable bacteria within a bacterial consortium (direct control of bacteria). For example, introduction of bacteria with high degradation activities into an activated sludge process will enhance the breakdown of recalcitrant compounds. Furthermore, extensive researches on the application of bioaugmentation have been carried out for removal of nutrients, stabilization of treatment performance, reduction of excess sludge production, control of abnormal foaming, improvement of sludge settling ability.

Figure 0.1 illustrates the general concept of bioaugmentation to the activated sludge process and one possible strategy is shown in the schematic flow chart in Fig. 0.2. The bioaugmentation strategies need synthesized knowledge of wastewater treatment engineering, bacteriology, genetics, and microbial ecology. First, problems which cannot be solved by existing processes and their bacterial aspects are surveyed, and desirable bacterial characters are presumed present. Selection and breeding of desirable bacteria for bioaugmentation involve adaptation, natural selection, and genetic engineering techniques. Natural bacterial strains can be used, but the breeding of new genetically engineered microorganisms (GEMs)

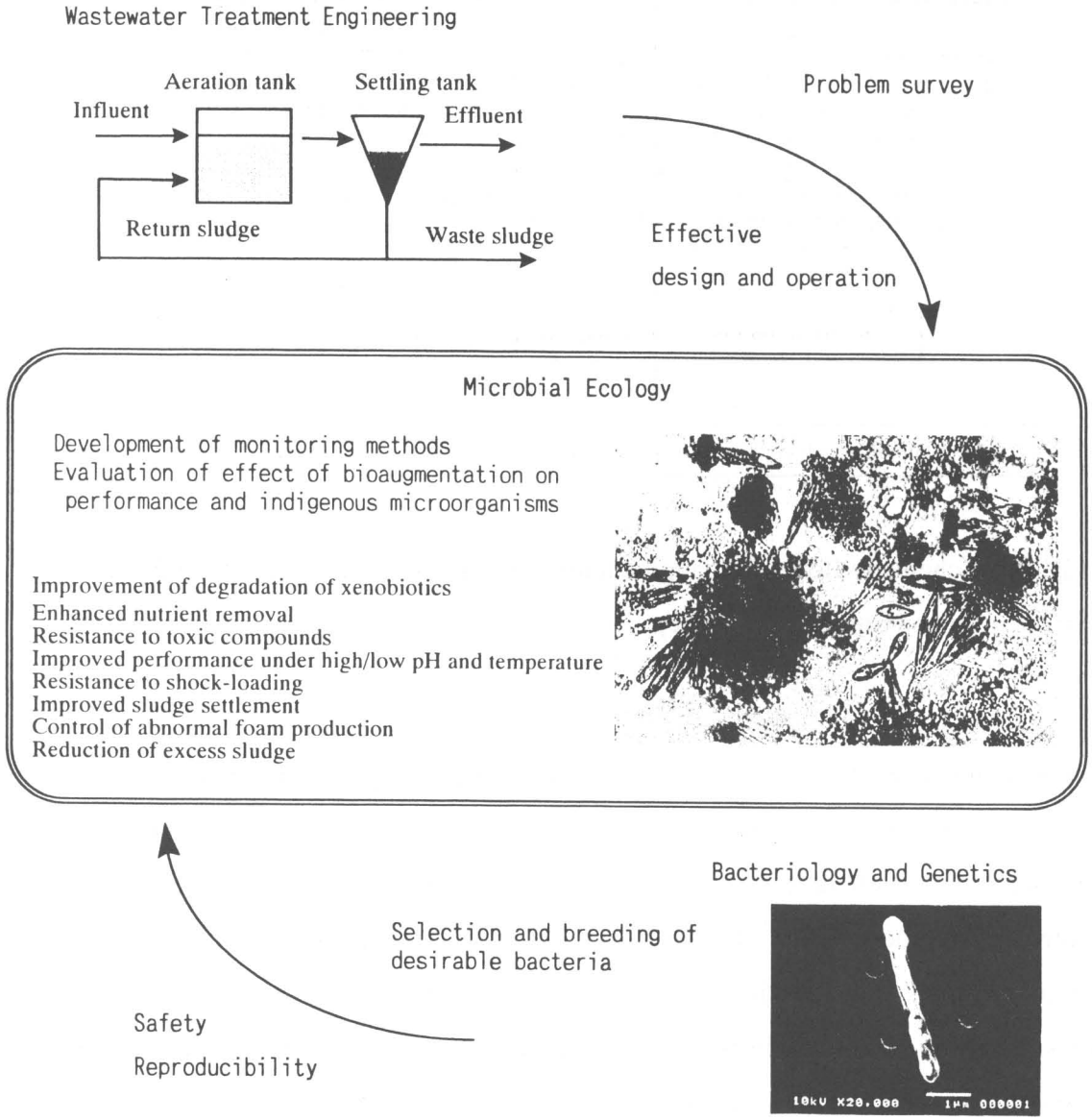

Fig. 0.1. General concept of bioaugmentation to activated sludge.

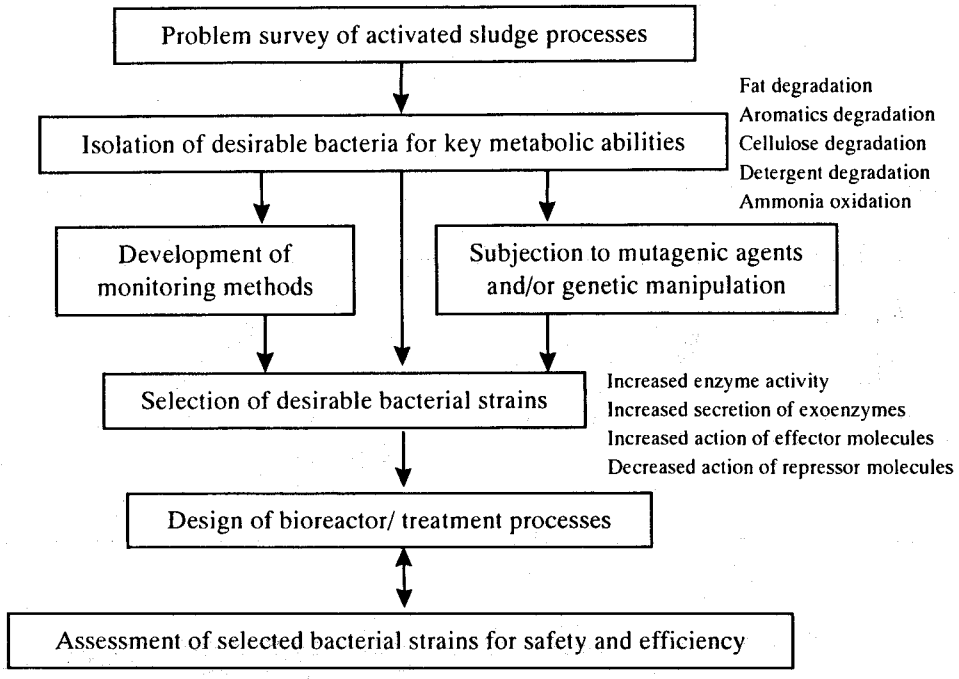

Fig. 0.2. Schematic flow of bioaugmentation to activated sludge (Fujita and Ike, 1994; Stephenson and Stephenson, 1992).

with the potential for the enhanced activity can also be very promising. Breeding of GEMs is carried out step-by-step with close adherence to legal regulations and guidelines for the field application. The resultant bacterial strains are tested in laboratories and fields for safety, efficacy, and reproducibility. A prerequisite for any bioaugmentation program is to have sensitive, reliable, and selective systems available to detect the introduced bacteria in the process. There are two monitoring strategies available: quantitative, where the introduced bacterial strain is enumerated, and qualitative, where the bacterial strain is monitored on the basis of presence or absence only. Evaluation of the bacterial strains should include assessments of the existing condition of the purpose and effect on the indigenous organisms. The pilot tests, which simulated actual treatment processes, should be performed afterwards for the selection, development, synthesis, and optimization of the treatment processes. Public opinion must also be taken into account (Lindow et al., 1989; Pikup et al., 1991; Tiedje et al., 1989).

However, the introduction of desirable bacteria into activated sludge to enhance the

removal of pollutants has not been yet widely applied. This is due to the fact that the behavior of the introduced bacteria has not been fully studied and is less predictable and controllable than the direct physical or chemical destruction of pollutants. From literature reviews (Limbergen and Verstraete, 1998; Espinosa and Stephenson, 1996; Stephenson and Stephenson, 1992), most of the bioaugmentation studies concluded that any apparent effect was not observed. Table 0.2 shows possible reasons for the failure of bioaugmentation. On the whole, the successful bioaugmentation requires at least that introduced bacteria survive in the process enough to exhibit their desirable metabolic activities. In order to assess the feasibility of their uses in the processes, intensive investigation of their behavior is necessary.

The behavior of the introduced bacteria has been made clear by degrees as results of laboratory experiments (Erb et al., 1997; Fujita et al., 1994a; Heitkamp et al., 1993; Kanagawa and Mikami, 1995; McClure et al., 1989, 1991; Nüblein et al., 1992; Selvaratnam et al., 1997; Wand et al., 1997; Watanabe et al., 1998). However, those experiments were carried out on a case-by-case basis, and those data could not always be applicable to predict the behavior of introduced bacteria under other conditions. For evaluating the effects of bioaugmentation on the treatment performance, it is primarily important to make clear the general behavior of the

Table 0.2. Possible reasons for failure of bioaugmentation to activated sludge (Stephenson and Stephenson, 1992)

1) Concentrations of target pollutants may be too low to support the growth of the introduced bacteria or those use other substrates in the process rather than the pollutants.
2) The process may contain inhibitory substances or be operating under unsuitable conditions, such as pH and temperature.
3) Competition with indigenous bacteria causes growth inhibition of the introduced bacteria.
4) The introduced bacterial population is too few to change the treatment performance.
5) The introduced bacteria are not put into the micro habitats where it can effectively degrade the target pollutants.

introduced bacteria. Development of a mathematical model describing the behavior of introduced bacteria in activated sludge would provide understanding how operational parameters of the process affect their behavior and how long they can ultimately survive in given conditions

The target of this study is to make clear the general behavior of introduced bacteria in the activated sludge process by means of developing a dynamic model. The schematic flow of this study is shown in Fig. 0.3. In Chapter 1, an experimental case study of an activated sludge process bioaugmented with a genetically engineered bacterium was carried out. From the results, it was confirmed that the effect of bioaugmentation depends on the survival of the introduced bacteria. In Chapter 2, operational factors of the process affecting the behavior of the introduced bacteria were studied and a concept for its modeling was proposed. In Chapters 3 and 4, physicochemical and biological important factors affecting the behavior of the introduced bacteria were studied in detail and sub-models for the modeling were developed. In Chapter 5, finally, the two sub-models were put into together and a dynamic model which can

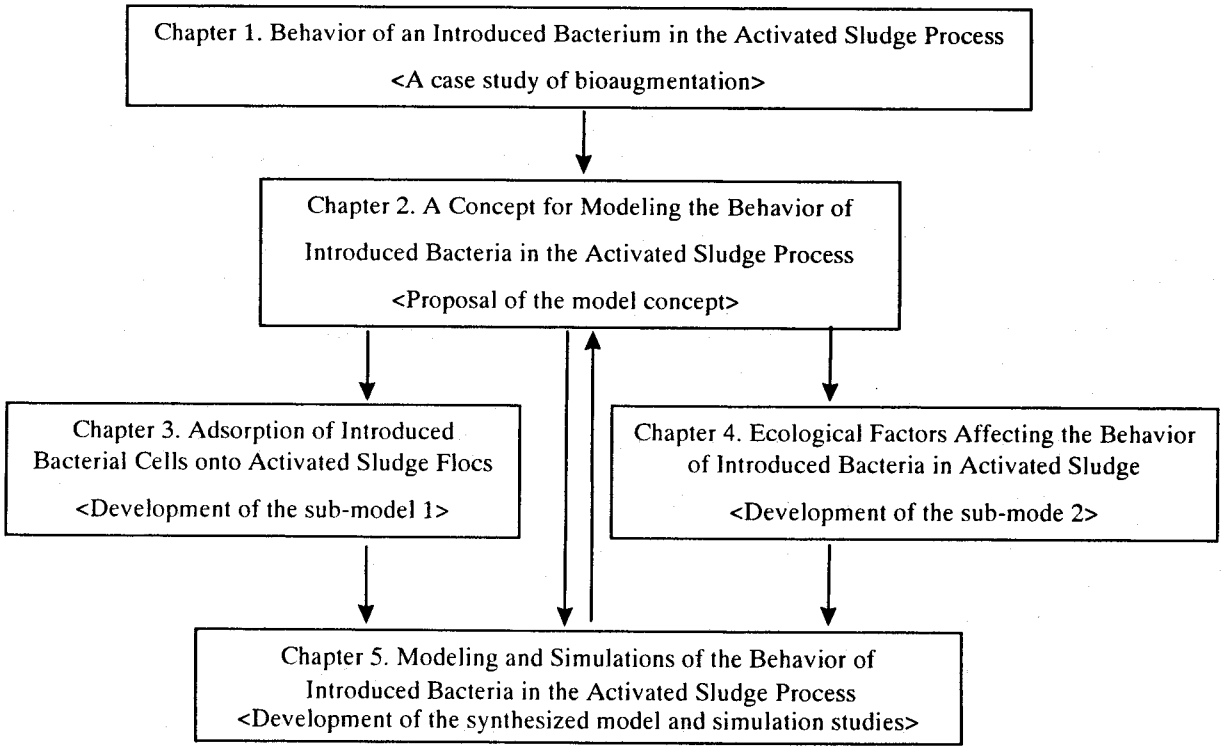

Fig. 0.3. Schematic flow of this study.

properly describe the behavior of introduced bacteria in activated sludge was developed, and potentials and limitations of bioaugmentation were discussed with simulation studies.

Chapter 1. Behavior of an Introduced Bacterium in an Activated Sludge Process

1-1. INTRODUCTION

First, as a case study of the bioaugmentation, the performance of a sequencing batch activated sludge reactor (SBR) bioaugmented with a genetically engineered microorganism (GEM) was investigated. Phenol was chosen as a model target pollutant because it is contained in wastewaters produced in a variety of industries and a highly toxic or inhibitory substance with microbiocidal properties. The influx of a high concentration of phenol into an activated sludge process seriously damages bacterial activity if it is not previously acclimated to phenol, and as a consequence the treatment performance deteriorates drastically. For example, decreases in organic carbon removal and increases in the sludge volume index (SVI) caused by the influx of phenol have been well documented (Bieszkiewicz and Fiutowska, 1991; Lund and Cabrera, 1986; Okada et al., 1991; Sudhani et al., 1991; Watanabe et al., 1996).

For this reason, phenolic wastewater has been treated for many years using well-acclimated activated sludge processes, with special attention being given both to their design and operation. SBRs are widely used for treating a variety of industrial wastewaters containing hazardous chemicals, since their performance is relatively little affected by fluctuations in the quantity and/or quality of the influent (Cardinal and Stenstrom, 1991; Hess et al., 1990, 1993; Okada et al., 1991; Wilder et al., 1991). However, when SBRs are applied to the treatment of phenol-containing wastewater, the introduced bacterial population will inevitably be exposed to a higher initial concentration of phenol than in a continuous flow process, as a result of which its toxic effect may reduce the effectiveness of the treatment performance.

In this chapter, the performance of a SBR bioaugmented with a GEM, *Pseudomonas putida* BH (pS10-45), which was designed to be able to efficiently degrade phenol at high concentrations was investigated with the aim of improving the treatment of phenol. The effects of the GEM-introduction on the treatment performance were examined in comparison with that of a control SBR which was not introduced with the GEM, and behavior of the GEM was monitored using a selective medium and the relationship between its behavior and performance of the SBR was discussed.

1-2. MATERIALS AND METHODS

Breeding of the GEM *P. putida* BH (pS10-45) The phenol-degrading bacterium *P. putida* BH (Hashimoto and Fujita, 1987), isolated from a phenol-acclimated activated sludge, was used as a host strain for genetic manipulation. The recombinant plasmid pS10-45 (Fujita et al., 1995) was constructed by cloning a DNA fragment containing the whole phenol operon (all the genes related to phenol degradation) from *P. putida* BH into a cosmid vector, pVK100 (Knauf and Nester, 1982), that gives its host resistance to kanamycin (Km). Plasmid pS10-45 was then re-introduced into the original *P. putida* BH strain by tri-parential mating with a helper plasmid, pRK2013 (Figurski and Helinski, 1979), to enhance its phenol-degrading activity.

Phenol degradation by *P. putida* BH (pS10-45) in pure culture A preliminary experiment was conducted to ascertain the enhanced phenol-degrading activity of the constructed GEM *P. putida* BH (pS10-45). The parent strain *P. putida* BH and the GEM were inoculated into LB medium (Sambrook et al., 1989) with or without 50 mg/*l* Km, and cultivated for 12 h as a pre-culture. One milliliter of the pre-culture broth was then transferred into 100 ml of new medium and incubated to the mid-log phase under the same conditions. Cells were harvested by centrifugation (10,000×*g*, 10 min., 4°C), washed with 5 mg/*l* sodium tripolyphosphate (pH 7.2), and suspended at an approximate turbidity of 1.0 at 660 nm in a basal salt medium (Fujita et al., 1995) supplemented with phenol at initial concentrations of 120, 200, or 500 mg/*l*. During incubation on a rotary shaker (100 rpm, 25 °C) the phenol concentration in the culture broth was periodically monitored by high-performance liquid chromatography (HPLC) (Fujita et al., 1995).

Model activated sludge process (SBR) A model activated sludge process was simulated by a shake culture cultivated in a sequencing batch mode (SBR) as follows. Seed activated sludge, which had been adapted to a synthetic wastewater for more than 5 years without any experience of exposure to phenol, was incubated in a 300-ml Erlenmeyer flask (working volume, 100 ml; mixed liquor suspended solids (MLSS) = 1700 mg/*l*) on a rotary shaker at 100 rpm and 25°C. After cultivation for 1 d, 20 ml of the mixed liquor was withdrawn as excess sludge, and the rest was allowed to settle for 30 min. This was followed by the discharge of 60 ml of clear supernatant as the effluent. Synthetic wastewater (80 ml) was then added up to the original volume (100 ml) as the influent. These operations were repeated daily regardless of the treatment performance. Under these basic conditions, the sludge retention time (SRT) and hydraulic retention time (HRT) were defined as 5 d and 1.25 d, respectively. Basically, the chemical composition of the synthetic wastewater fed to the model SBR was as follows: meat extract 400 mg, peptone 600 mg, urea 100 mg, $Na_2HPO_4 \cdot 12H_2O$ 83.6 mg, KH_2PO_4 13.6 mg, NaCl 30 mg, KCl 14 mg, $CaCl_2 \cdot 2H_2O$ 18.54 mg, $MgSO_4 \cdot 2H_2O$ 20.5 mg, and tap water 1 *l*. The synthetic wastewater contained approximately 440 mg/*l* dissolved organic carbon (DOC). For the phenol treatment experiments, phenol was added to the synthetic wastewater (influent) at definite concentrations.

Phenol treatment experiments The GEM *P. putida* BH (pS10-45) grown to the mid-log phase in 100 ml of LB medium was harvested by centrifugation (10,000 × g, 10min., 4°C), washed twice with 5 mg/*l* sodium tripolyphosphate, suspended in the synthetic wastewater, and then introduced directly into the model SBR at about 10^8 CFU/ml on day 0 (GEM-introduced SBR). Phenol contained in the synthetic wastewater was supplied to the SBR as a shock loading of about 500 mg/*l* on days 0 (1st shock loading), 7 (2nd shock loading), and 14 (3rd shock loading) (shock-loading study). After the experiments had started, phenol, DOC, and suspended solids (SS) in the effluent and the SVI of the mixed liquor were periodically measured. Phenol was measured by HPLC (Fujita et al., 1995) and the DOC was determined using a TOC-500 analyzer (Shimidzu, Kyoto) for filtered samples with 1.0-μm-pore-size glass microfibre filters (Whatman, Maidstone, England). The SVI (MLSS and SV_{30}) and SS were measured according to the methods set out in JIS (Japan Industrial Standards) K0102 with the modification that, because of the restricted working volume, the SV_{30} values were measured with a 100-ml graduated cylinder instead of a 1-*l* sample as is generally used. The same model SBR was also operated without GEM-inoculation for comparison (control SBR).

Enumeration of bacteria The populations of the GEM *P. putida* BH (pS10-45), indigenous phenol degraders, and total heterotrophic bacteria in the SBRs (excess sludge) were monitored by the plate count technique during the phenol treatment experiments. *P. putida* BH (pS10-45) was enumerated by a basal salts medium containing 500 mg/*l* sodium benzoate as the sole carbon source with 50 mg/*l* Km. In this study, bacterial counts on CGY medium (Pike et al., 1972) were defined as total heterotrophic bacteria. Bacteria enumerated on the basal salts medium containing 400 mg/*l* phenol as the sole carbon source was defined as total phenol degraders. Indigenous phenol degraders were defined as the counts of total phenol degraders minus the counts of the GEM. Excess sludge samples (5 ml) were diluted 10-fold with 5 mg/*l* sodium tripolyphosphate, treated with a sonicator (UD201: Tomy, Tokyo) to disperse the bacterial cells from the sludge flocs, and plated onto each medium. Plates were incubated at 30°C for 2 d to count the GEM, and for 7 d for both the total heterotrophic bacteria and total phenol degraders.

1-3. RESULTS

Breeding of the GEM *P. putida* BH (pS10-45) Introduction of pS10-45 into *P. putida* BH resulted in the construction of the GEM *P. putida* BH (pS10-45). The GEM was designed to possess a few or several copies of the phenol catabolic operon per cell on the multi-copy-number plasmid pS10-45 in addition to the one copy coded on the chromosome, although the original wild strain *P. putida* BH possessed only one copy of the operon on its chromosome. In other words, the phenol catabolic pathway of *P. putida* BH should have been amplified by the genetic manipulation.

Phenol degradation by the constructed GEM in a pure culture is shown in Fig. 1.1 in comparison with that by the original strain *P. putida* BH. The errors in the HPLC measurement for phenol were less than $\pm 5\%$. The GEM exhibited much faster phenol removal than the original strain at initial concentrations of 200 and 500 mg/*l* although no apparent difference was observed in the degradation at an initial concentration of 120 mg/*l*. A lag period was observed in the time course of each degradation, which seemed to be indicative of the time necessary for sufficient induction of phenol hydroxyrase (Fujita et al., 1993a). The rate of each degradation in the lag period (k_1) and the post-lag period (k_2) was estimated by zero-order kinetics with good agreement ($r > 0.96$). The lag period (T_L) was estimated as the crossing point of the dual degradation lines. The T_L of the original strain at an initial

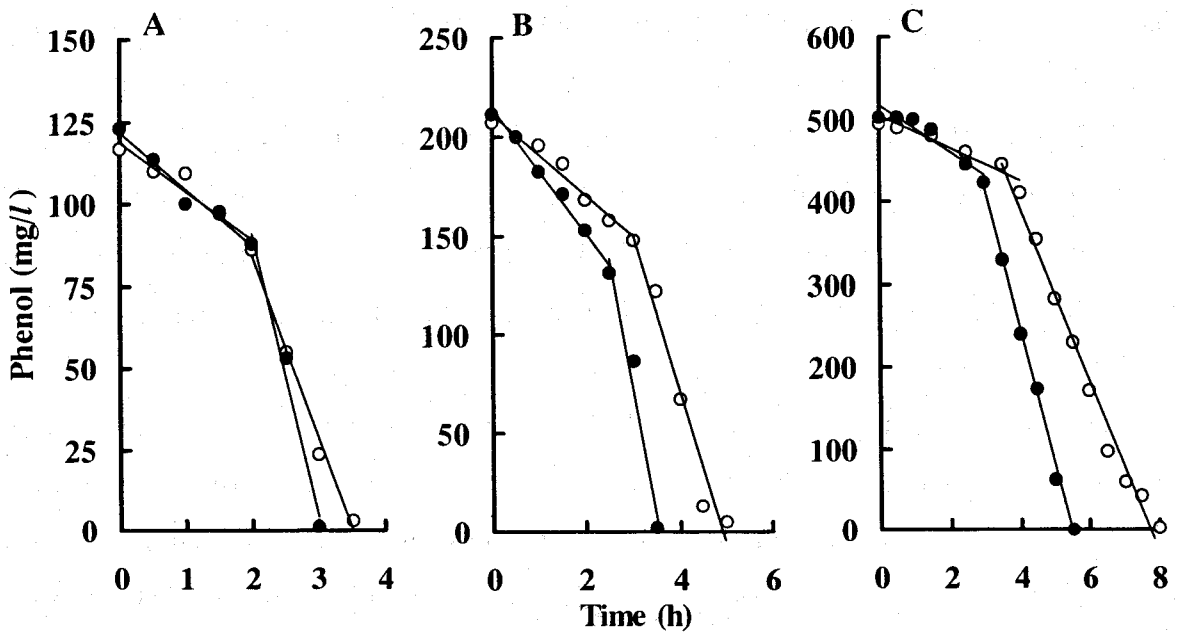

Fig. 1.1. Phenol degradation by the GEM *P. putida* BH (pS10-45). Time courses of phenol degradation at initial concentrations of 120 (A), 200 (B), and 500 mg/*l* (C) by the GEM (●) are shown in comparison with that by the original strain *P. putida* BH (○). Dual zero-order kinetics were fitted to the experimental data. The crossing points of the dual regression lines and the slopes of the lines signify the lag period for sufficient induction of phenol hydroxyrase and the zero-order degradation rate constant, respectively.

Table 1.1. Dual zero-order kinetics of phenol degradation by the GEM and the original strain

Initial phenol	Original strain *P. putida* BH			GEM *P. putida* BH (pS10-45)		
concentration (mg/*l*)	T_L	k_1	k_2	T_L	k_1	k_2
120	1.9	1.5×10^{1}	5.6×10^{1}	2.1	1.7×10^{1}	8.7×10^{1}
200	3.1	2.0×10^{1}	9.2×10^{1}	2.6	3.2×10^{1}	1.3×10^{2}
500	3.7	2.0×10^{1}	1.1×10^{2}	2.9	2.8×10^{1}	1.7×10^{2}

k_1, zero-order degradation rate constant during the lag period (mg/*l*/h); k_2, zero-order degradation rate constant after the lag period (mg/*l*/h); T_L, lag period (h).

concentration of 500 mg/*l* was 3.7 h, while that of the GEM was only 2.9 h. Thus, the zero-order degradation rate constants of the GEM in both the lag and post-lag periods were approximately 1.5 times higher than those of the original strain, as shown in Table 1.1. No phenol metabolite (catechol, *cis*-,*cis*-muconate, 2-hydroxymuconic semialdehyde, etc.) was detected in either culture. Additional experiments on phenol degradation by the activated sludge introduced with the GEM and the original strain were carried out. Similar results to those shown in Fig. 1.1 were obtained, *i.e.*, the GEM-introduced SBR showed higher phenol degradation than the SBR inoculated with the original strain (data not shown).

Results of the shock-loading study Phenol removal by the GEM-introduced SBR for each shock loading is summarized in Table 1.2 in comparison with that in the control experiment. Phenol removal was calculated from the phenol concentrations in the influent and effluent at the end of each batch cycle. Figure 1.2 shows the daily changes in DOC removal, SS in the effluent, and the SVI of the mixed liquor for the GEM-introduced and control SBRs. The errors in these measurements were less than ± 10 %.

In comparison with the control SBR, the GEM-introduced SBR could cope with phenol better after the 1st and 2nd shock loadings. However, phenol removal by the GEM-introduced SBR after the 3rd shock loading was considerably lower than that of the control. Over the experimental period, phenol removal by the control SBR rose from 25.2 to 50.3%, that by the GEM-introduced SBR dropped from 99.6 to 30.6%, indicating that the control SBR gradually acclimated to phenol while the effect of the inoculation of the GEM was gradually lost. DOC removal in both the control and GEM-introduced SBRs was normally maintained above 90%; however, DOC removal dropped on days 1, 8, and 15 due to incomplete treatment of the phenol fed as shock loadings. The DOC removal in the GEM-introduced SBR after the 1st and 2nd phenol shock loadings was considerably higher than that of the control, reflecting its higher phenol removal efficiency. The SS values in the effluents of both SBRs were generally maintained below 70 mg/*l*; however in the GEM-introduced SBR on the day 1 the SS was very high, rising to about 250 mg/*l*. This large increase in SS seemed to be due to the washing out of GEM cells that were introduced on day 0 but were unable to become sufficiently attached to flocs within 24 h. The daily changes in the SVI, show that the settling capability of the control activated sludge grew more unstable and worsened gradually over the period during which the three phenol shock loadings were given, while the GEM-introduced activated sludge maintained relatively good settling capability.

Table 1.2. Comparison of phenol removal (%) by the GEM-introduced and control SBRs

Shock loading	Control SBR	GEM-introduced SBR
1st (day 0)	25.2	99.8
2nd (day 7)	27.3	60.0
3rd (day 14)	50.3	30.6

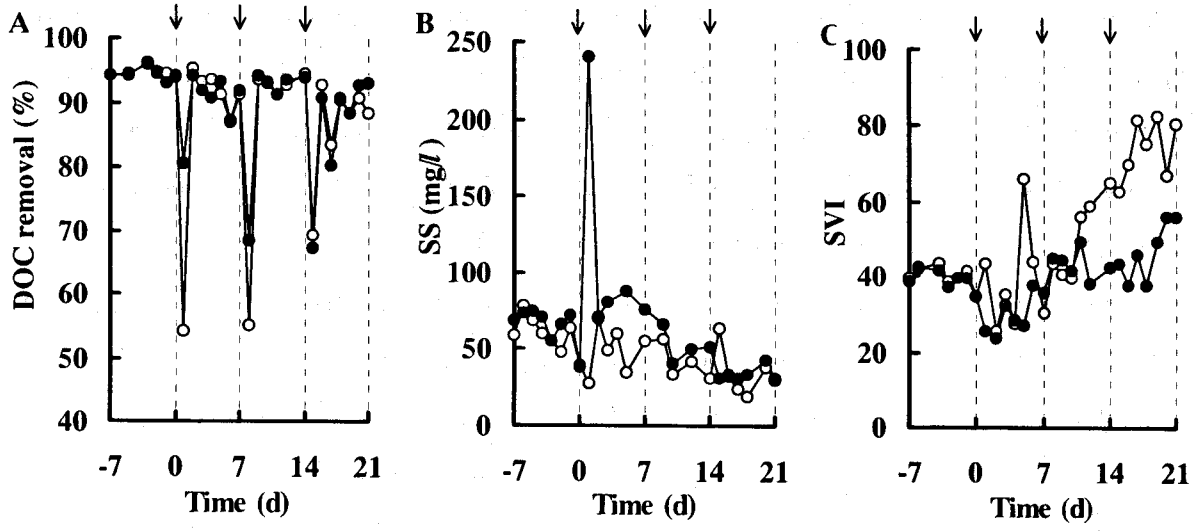

Fig. 1.2. Comparison of treatment performance of the GEM-introduced (●) and control (○) SBRs in the shock-loading study. Time courses of DOC removal (A), SS in the effluent (B), and the SVI of the mixed liquor (C) are shown. Phenol shock loadings (500 mg/*l*) were given on days 0, 7, and 14 (arrows). On day 0, the GEM was introduced into the GEM-introduced SBR at 10^8 CFU/ml.

The population dynamics of the introduced GEM and indigenous phenol degraders in the SBRs are shown in Fig. 1.3. On the selective medium for the GEM, the viable counts of the activated sludge samples in the control SBR were below 10^1 CFU/ml. To evaluate the stability of the plasmid pS10-45 in the host strain *P. putida* BH, the activated sludge samples in the GEM-introduced SBR were plated onto the selective medium or the medium without Km (incubated at 28℃ for 2 d), and the segregants (host cells without plasmid pS10-45) were estimated. The results suggested that the percentage of segregants was within 5% throughout the experimental period (data not shown). The phenotypes and morphologies of several colonies formed on the selective medium were investigated, and all the colonies tested

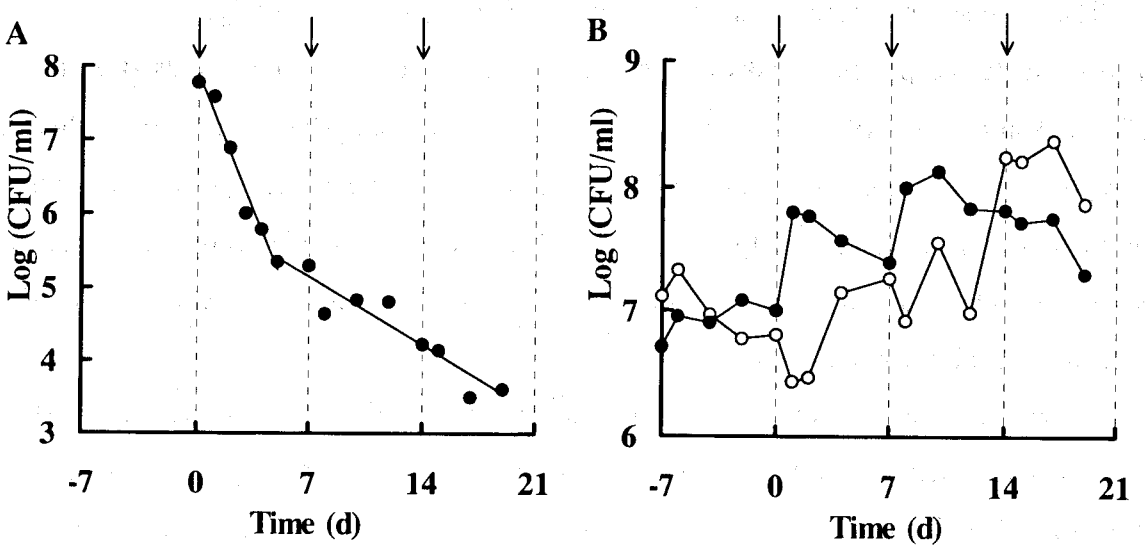

Fig. 1.3. Population dynamics of the GEM (A) and indigenous phenol degraders (B) of the GEM-introduced (●) and control (○) SBRs in the shock-loading study. Phenol shock loadings (500 mg/*l*) were given on days 0, 7, and 14 (arrows). On day 0, the GEM was introduced into the SBR at 10^8 CFU/ml. The T_{90} values for the GEM in the rapid declining and slow declining phases were estimated to be about 1.9 and 7.6 d, respectively.

showed the same phenotypes and morphologies as those of the introduced GEMs. Therefore, the selective medium used seemed to be specific to enumerate only the GEM.

The population of the GEM dropped from 10^8 to 10^4 CFU/ml over 14 d. The decline seemed to be divided into two phases: the GEM population decreased rapidly over the first 5-7 d (rapid declining phase), after which fell gradually (slow declining phase). Similar biphasic survival patterns have been commonly observed in previous studies (Fujita et al., 1994a; McClure et al., 1991; Nüblein et al, 1992). The T_{90} values (T_{90} being defined as the time necessary for a decrease of 90% in the GEM population) in the rapid declining and slow declining phases were estimated to be about 1.9 and 7.6 d, respectively. GEM-introduction had a considerable effect on the behavior of indigenous phenol degraders, which increased gradually in both SBRs, but more rapidly in the GEM-introduced SBR than in the control SBR, through the experimental period. However, the final population size in the GEM-introduced SBR was a little smaller than that in the control SBR. The total population of heterotrophic bacteria in both SBRs were kept stable at about 10^9 CFU/ml during the experimental period (data not shown).

1-4. DISCUSSION

There are some reports on the behavior of GEMs in activated sludge processes, which aim to evaluate the potential risks of their accidental release into open fields (Gealt et al., 1985; Heitkamp et al., 1993; Mancini et al., 1987; Wand et al., 1997). In contrast, there are few reports on the application of GEMs to activated sludge processes with the aim of improving or enhancing the performance of such processes, although wastewater treatment processes, which usually have disinfection equipment, are envisaged as acceptable open fields for the intentional introduction of GEMs.

Enhanced phenol removal as a result of GEM-introduction was observed in the shock-loading study, though the effect was gradually lost in line with the decrease in the GEM population. This indicates that the GEM was effective in removing an extremely high concentration of phenol. On the other hand, as noted in other studies (McClure et al., 1991; Mohamed et al., 1990; Nüblein et al, 1992), survival of the GEM at a significantly high level appeared to be a requisite for maintaining high phenol-removal efficiency. Therefore, for successful bioaugmentation, it is necessary to develop strategies for improving the survival of introduced bacteria in activated sludge.

There is very little information in the literature concerning the apparent effects of introduced bacteria on the behavior of indigenous bacteria. In fact, the population dynamics of indigenous and introduced bacteria in an equal or close niche have hardly been monitored in previous bioaugmentation studies, and the consequent lack of data on such bacterial populations has hampered clear elucidation of the effects of bioaugmentation.

The decline of the GEM-survival was divided into two phases, rapid declining phase and slow declining phase. The T_{90} value means the specific decrease rate of the GEM in activated sludge. The theoretically expected specific decrease rates of the GEM due to effluent discharge and sludge wastage are only 0.8 d^{-1} (= HRT^{-1}) and 0.2 d^{-1}(= SRT^{-1}), respectively. This indicates that the introduced GEM in activated sludge encountered other strong selective pressures which may include predation by protozoa, competition with indigenous bacteria, and fatal environmental effects. One possible way is to prevent the GEM cells from being washed out from activated sludge processes in the rapid declining phase by employing cell immobilization techniques (Fujita et al., 1991; Muyima et al., 1995) or using a floc-forming bacterium as a host in genetic manipulation (Fujita et al., 1991; McClure et al., 1991). Proper feeding of target pollutants, phenol in this study, may also be advantageous in placing selective pressure on the GEM. Additions of supplemental substrates which are not target

pollutants can also be an important means of providing selective pressure (Fujita et al., 1994a; Hess et al., 1990).

Another interesting and novel finding in this study was that introduction of the GEM affected the population dynamics of indigenous phenol degraders in the activated sludge, *i.e.*, acclimation of the activated sludge to phenol. In the shock-loading study, phenol removal by the control SBR gradually rose, accompanied by a gradual increase in the population of indigenous phenol degraders, while the phenol-removal capability of the GEM-introduced SBR gradually dropped with a decrease in the GEM population. Although the indigenous phenol degraders in the GEM-introduced SBR also increased, their population at the end of the shock-loading study was smaller than that in the control SBR.

1-5. SUMMARY

A genetically engineered microorganism (GEM) *P. putida* BH (pS10-45), which was designed to exhibit higher phenol-degrading activity than the wild strain, was introduced into a model activated sludge process to improve its phenol-treatment performance as a case study of the bioaugmentation. The model activated sludge process was operated as a 24-h-cycle sequencing batch reactor in a shake flask, into which phenol-containing wastewater (500 mg/*l*) was fed in the form of a shock loading three times at 7-d intervals. The GEM introduced into activated sludge showed a biphasic pattern on their populations, *i.e.,* the rapid declining phase and the slow declining phase. After the shock loadings, the phenol-removal efficiency of the GEM-introduced activated sludge was much enhanced in comparison to that of a control process without GEM-inoculation for as long as the GEM population survived at a relatively high level.

Chapter 2. A Concept for Modeling the Behavior of Introduced Bacteria in the Activated Sludge Process

2-1. INTRODUCTION

In Chapter 1, the introduced bacterial strain in activated sludge showed low ecological stability; their population declines rapidly (rapid declining phase), after which they remain relatively stable or decline slowly (slow declining phase). Ecological stability refers to the length of time introduced bacteria can survive and express their useful activities in the mixed bacterial community of the activated sludge process. It also refers to the population size introduced bacteria can maintain there. Even if a desirable bacterial strain with a high recalcitrant compound degradation rate is selected or bred, it may soon disappear dramatically from the process if it has low ecological stability, and the treatment efficiency will thus be little improved. For predicting or evaluating the effects of bioaugmentation, it is primarily important to make clear the operational factors of the process the general survival-mechanism of the introduced bacteria.

In this chapter, survival experiments of two genetically engineered bacteria, *Pseudomonas putida* BH (pBH500) and *Escherichia coli* C600 (pBH500), were carried out under various operational conditions of an activated sludge process where no specific selective pressure was present. From the experimental results, a fundamental survival mechanism of the introduced bacteria were discussed. Development of an appropriate model describing the behavior of the introduced bacteria in the process would provide understanding of how operational parameters of the process affect their behavior and how long they can ultimately survive in given conditions.

2-2. EXPERIMENTALS

Materials and methods *P. putida* BH (Hashimoto and Fujita, 1989) and *E. coli* C600 (Appleyard, 1954), which is a bacterial host most usually used in genetic engineering, were introduced into activated sludge. Recombinant plasmid pBH500 (Fujita et al., 1991) was introduced into both strains to give specific selective markers resistance to streptomycin and expression of a catechol 2,3-oxygenase gene (*pheB*). Colonies expressing *pheB* quickly turn yellow with 0.1mM catechol solution sprayed. The plasmid pBH500 was very stable in both host strains, and segregants hardly occurred (Fujita et al., 1994a).

The activated sludge process was simulated by a shake culture cultivated in a sequencing batch reactor (SBR) as shown Fig. 2.1. The chemical composition of the synthetic wastewater fed into the SBR was as follows: meat extract 400 mg, peptone 600 mg, urea 100 mg, $Na_2HPO_4 \cdot 12H_2O$ 83.6 mg, KH_2PO_4 13.6 mg, NaCl 30 mg, KCl 14 mg, $CaCl_2 \cdot 2H_2O$ 18.6 mg, $MgSO_4 \cdot 2H_2O$ 20.5 mg, and 1 litter of tap water. The seed activated sludge which had been adapted to the synthetic wastewater for more than 5 years was incubated in a 300-ml Erlenmeyer flask (working volume 100 ml, mixed liquor suspended solids = *c.a.* 2000 mg/*l*) on a rotary shaker at 100 rpm and 25 °C. After a one day cultivation, 5 ml of the mixed liquor

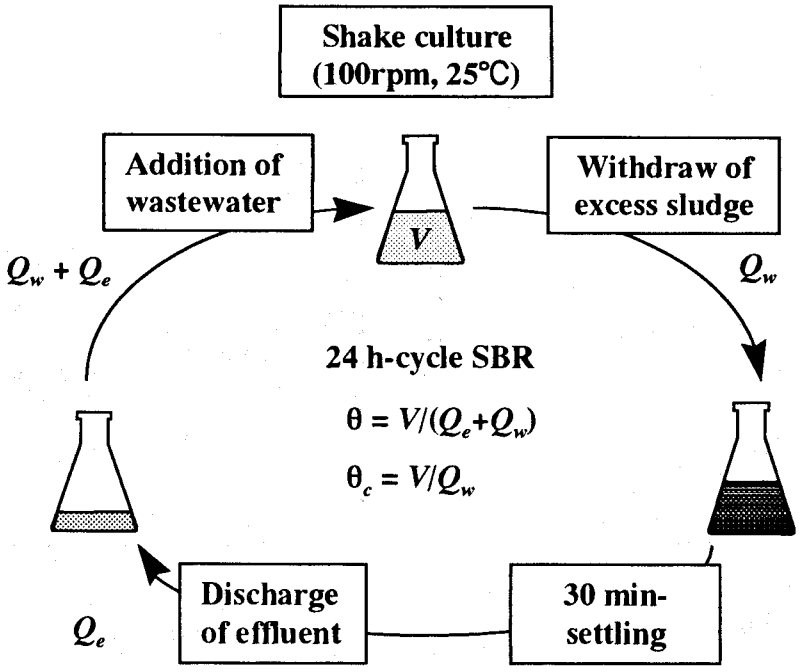

Fig. 2.1. Model activated sludge process simulated in a sequencing batch reactor mode.

suspended solids was withdrawn, and the rest was settled for 30 min, followed by the discharge of 75 ml of clear supernatant. Then, the synthetic wastewater (80 ml) was added up to the

original volume (100 ml) as influent. Such operations were repeated daily. Under this basic condition, the sludge retention time (SRT: θc) and the hydraulic retention time (HRT: θ) were defined as 20 d and 1.25 d, respectively.

Each bacterial strain, grown to the mid-log phase in 100 ml LB broth (Sambrook et al., 1989), was collected by centrifugation (10,000×g, 10min, 4℃), washed twice with 5 mg/l sodium tripolyphosphate buffer, suspended in the synthetic wastewater, and then introduced into the SBR. The viable counts of the introduced bacteria in the mixed liquor (excess sludge) and the effluent (supernatant) were periodically made by plate count technique with the selective media (Fujita et al., 1994a). The viable counts of heterotrophic bacteria in the mixed liquor and the effluent were also periodically made by plate count technique with CGY medium (Pike et al, 1972). Enumerations were made in triplicate.

Experimental results Fig. 2.2 shows typical results of the behavior of the introduced bacteria in the SBR (θ = 1.25 d, θc = 20 d). After a rapid decline in the number of the introduced bacteria was observed, their populations remained relatively stable. The numbers of *P. putida* BH (pBH500) and *E. coli* C600 (pBH500) contained in the effluent of the SBR were maintained at only about 1-30 and 1-10 % of these in the mixed liquor suspended solids,

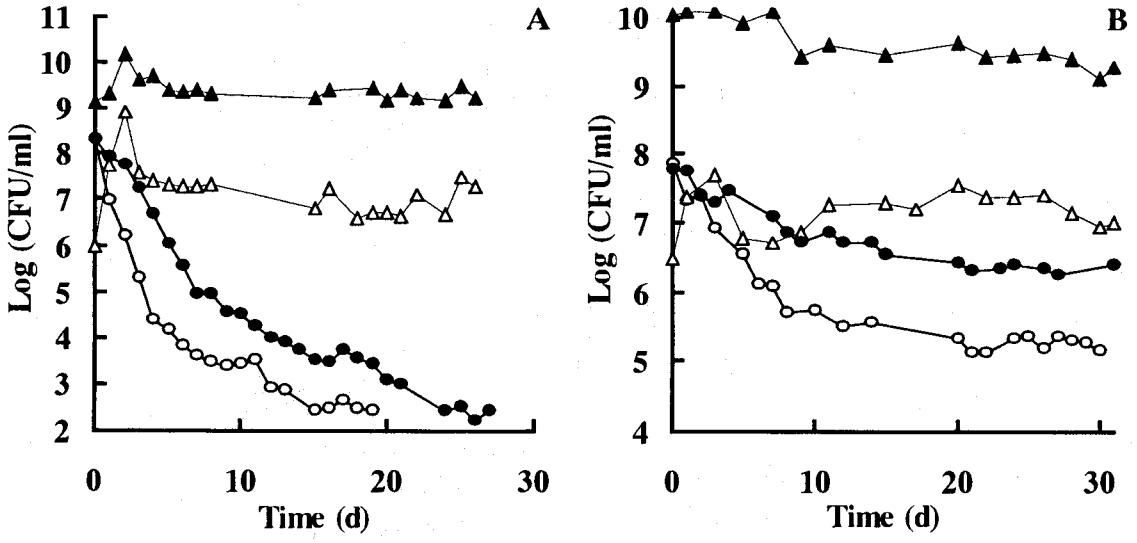

Fig. 2.2. Behavior of *P. putida* BH (pBH500) (A) and *E. coli* C600 (pBH500) (B) in the SBR (θ = 1.25 d, θc = 20 d). Viable counts of the introduced bacteria in activated sludge (●) and in effluent (○), and heterotrophic bacteria in activated sludge (▲) and in effluent (△) are shown.

respectively. These results indicate that their cells being washed out through the effluent were as many as those withdrawn as excess sludge.

The influence of the inoculum size of the bacteria and HRT and SRT of the SBR on their behavior is shown in Figs. 2.3 and 2.4. The biphasic behavior which consists of the rapid declining phase and the slow declining phase occurred in all experiments with different conditions. The larger the inoculum sizes, the introduced bacteria survived at higher levels in the SBR as shown in Figs. 2.3 (A) and 2.4 (A). Under larger HRT operations, the introduced bacteria survived at higher levels during the rapid declining phase as in Figs. 2.3 (B) and 2.4 (B), though the survival of *P. putida* BH (pBH500) in the slow declining phase got little influenced. The bacteria are in the suspended form soon after inoculation, therefore they can be easily withdrawn into the effluent. On the other hand, under smaller SRT operations, the introduced bacteria survived at higher levels in the slow declining phase, though the survival of both introduced bacteria during the rapid declining phase got hardly influenced as seen in Figs. 2.3 (C) and 2.4 (C). Small SRT operations make the bacteria with low growth rates washed out from the process and the diversity of indigenous bacterial flora decreases (Fujita and Ike, 1994), therefore, *P. putida* BH (pBH500) and *E. coli* C600 (pBH500) which have relatively high maximum specific growth rates may be increased.

These results indicate that the behavior of the introduced bacteria mainly depends on the initial adsorption of the cells onto flocs and their growth in flocs. In other words, the bacteria with the high adsorption rate onto and the high growth rate in flocs have a great possibility to survive at a high level. Some researchers also reported that the bacteria with the relatively high growth rate survived longer in sewage (Sinclair and Alexander, 1989), and that floc-forming bacteria showed higher survival levels (McClure et al., 1991; Watanabe and Hino, 1996). Immobilization techniques (Muyima and Cloete,1995) and additions of specific substrates (Kanagawa and Mikami, 1995; Hess et al., 1990, 1993; Watanabe et al., 1998) may be also reasonable strategies to enhance the survival of the bacteria.

2-.3 MODEL CONCEPT

Two sub-populations The biphasic survival pattern of the introduced bacteria in activated sludge was experimentally confirmed as a general phenomena. Also, it was demonstrated that HRT and SRT have a great influence on the behavior of the introduced bacteria in the process. These suggested that the bacteria in activated sludge consists of two sub-populations each of which has a different behavior.

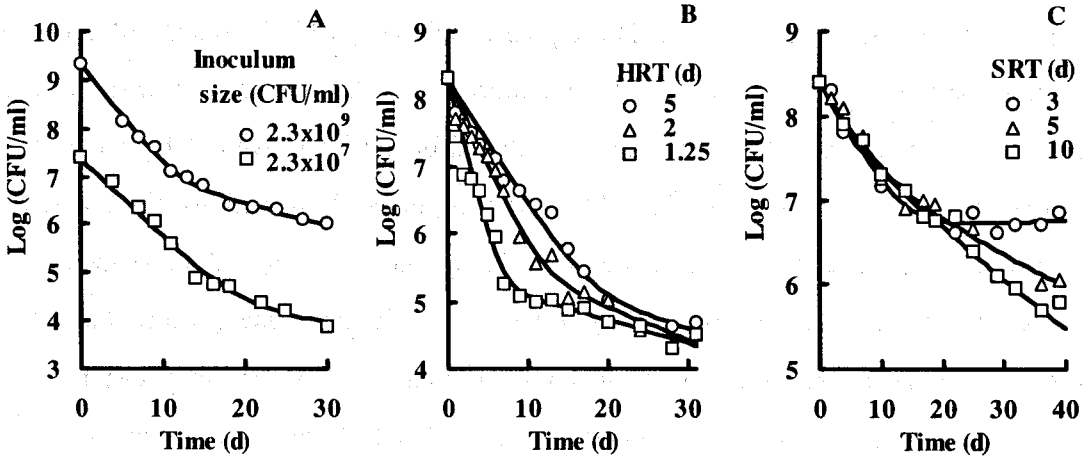

Fig. 2.3. Behavior of *P. putida* BH (pBH500) in the SBR under various operational conditions. The survival at two inoculum sizes (θ = 1.25 d, θc = 20 d) (A), at three HRTs (inoculum size = 2.0×10^8 CFU/ml, θc = 5 d) (B), and at three SRTs (inoculum size = 2.7×10^8 CFU/ml, θ = 1.25 d) (C) are shown.

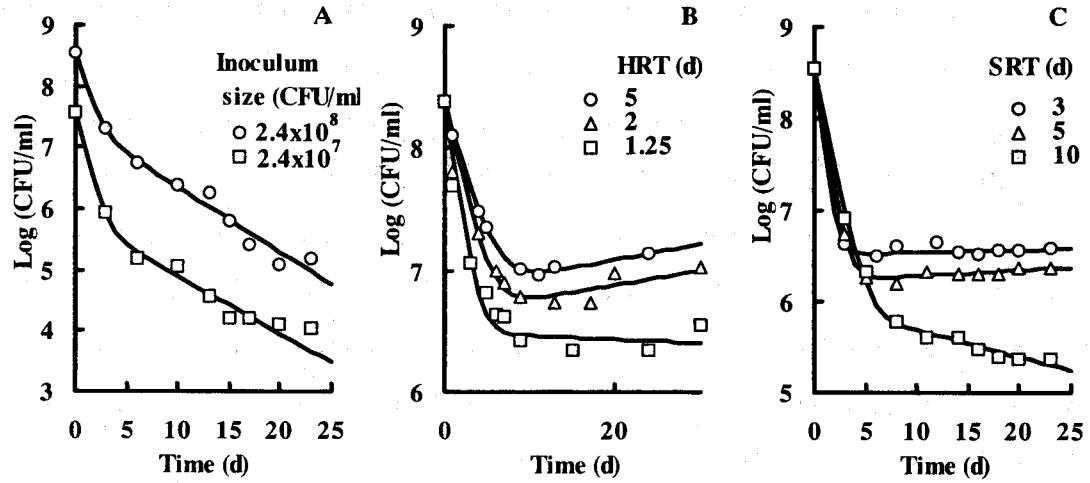

Fig. 2.4. Behavior of *E. coli* C600 (pBH500) in the SBR under various operational conditions. The survival at two different inoculum sizes (θ = 1.25 d, θc = 20 d) (A), at three HRTs (inoculum size = 2.4×10^8 CFU/ml, θc = 5 d) (B), and at three SRTs (inoculum size = 2.7×10^8 CFU/ml, θ = 1.25 d) (C) are shown.

As the primary concept for model development which describes the general survival mechanism of the bacteria in activated sludge, it was assumed the two sub-populations should be a sub-population of the bacteria adsorbed onto sludge flocs (X_f : Sub-population adsorbed onto flocs) and one of those in the suspended form or being detached from flocs (X_s : Sub-population in the suspended form). This concept is described in equation 2.1 and illustrated in Fig.2.5.

(Total population of an introduced bacterium in activated sludge) =

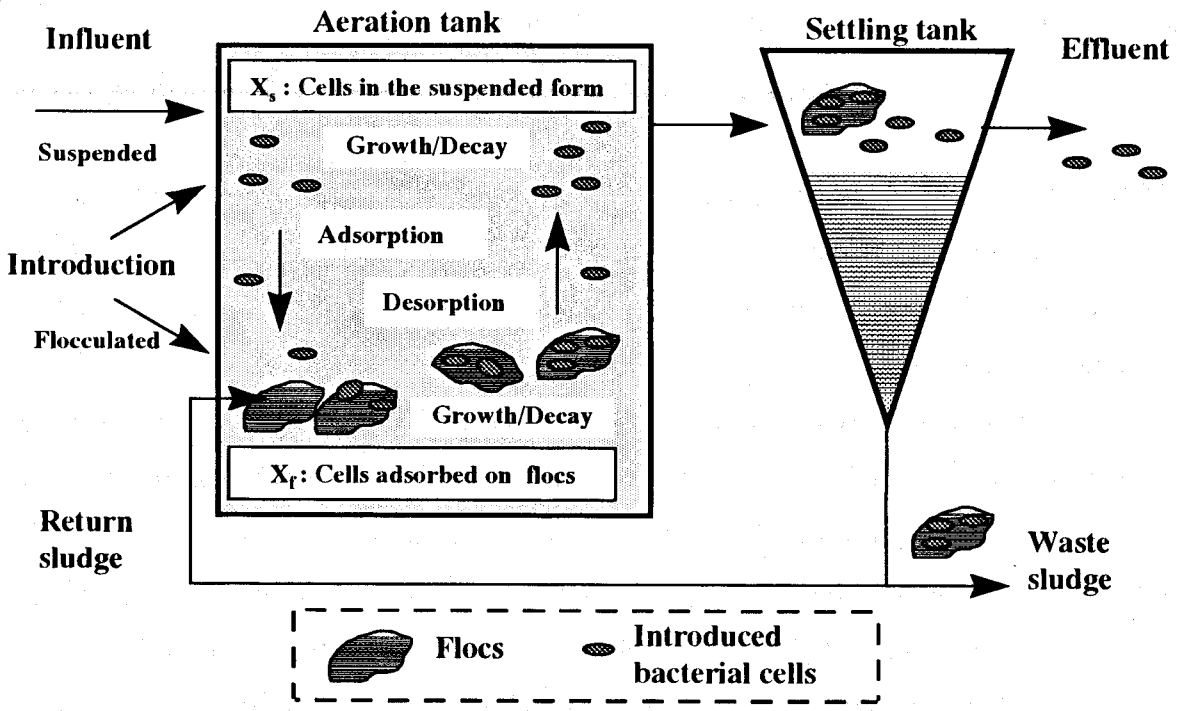

Fig. 2.5. Concept for modeling the behavior of introduced bacteria in the activated sludge process. The introduced bacteria in mixed liquor consist of two sub-populations, the adsorbed population onto flocs (X_f: Sub-population adsorbed onto flocs) and the suspended population (X_s: Sub-population in the suspended form), and mutual transfer between the sub-populations is caused by adsorption and desorption.

(Sub-population adsorbed onto flocs) + (Sub-population in the suspended form)

$$VX = V(X_f + X_s) \quad (2.1)$$

where V: volume of aeration tank, X: total population density of a introduced bacterium in activated sludge, X_f: sub-population density of $\mathbf{X_f}$, and X_s : sub-population density of $\mathbf{X_s}$.

Factors, such as growth and decay of bacteria, adsorption onto flocs, desorption from flocs, waste sludge rate, and effluent rate, should encourage or restrict their survival in activated sludge. The mass balances of $\mathbf{X_f}$ and $\mathbf{X_s}$ can be expressed as follows.

(Increase rate of $\mathbf{X_f}$) = (Inflow) - (Outflow) + (Growth) - (Decay)
+ (Adsorption of $\mathbf{X_s}$ onto flocs) - (Desorption of $\mathbf{X_f}$ from flocs) **(2.2)**

(Increase rate of $\mathbf{X_s}$) = (Inflow) - (Outflow) + (Growth) - (Decay)
- (Adsorption of $\mathbf{X_s}$ onto flocs) + (Desorption of $\mathbf{X_f}$ from flocs) **(2.3)**

Inflow In general, introduced bacteria in activated sludge seem to be introduced either of two forms. One is the suspended form and the other is the flocculated or immobilized form. The introduction in the suspended form or in a flocculated form means, respectively, the introduction of $\mathbf{X_s}$ or $\mathbf{X_f}$.

Recently, much research into the application of immobilization techniques to wastewater treatment have been carried out, and their high potential for improving the treatment performance and solving problems concerning solid-liquid separation in the secondary settling tank has been recognized (Fujita et al., 1991; Muyima and Cloete,1995; Sumino et al., 1992). A most important advantage of the use of immobilization techniques in wastewater treatment is to be able to maintain high biomass concentration, which is regarded as impossible to attain in a normal suspended biological process.

Outflow Activated sludge processes rely heavily on the ability of bacteria to flocculate, allowing a straightforward separation of the formed biomass and the effluent in the secondary settling tank (Sakai et al., 1985; Shimizu and Odawara, 1985; Tago et al., 1975; Tanaka et al., 1985). The sludge waste rate determines SRT affecting bacterial populations and diversity in the process (Fujita and Ike, 1994; Brown and Knapp, 1990). If secondary settling tanks ideally function, it can be assumed that outflows of $\mathbf{X_f}$ and $\mathbf{X_s}$ from the activated sludge process

occurred by sludge waste and discharge of effluent, respectively. For an efficient separation, the biomass must grow in flocs of 100-200 μm with a density grater than 1 g/cm (Verstraete and van Vaerenberg, 1986).

Recently, some research into the application of ultrafiltration membranes to wastewater treatment have been carried out. Porter (1990) reported on the coupling of a ultrafiltration membrane to an activated sludge process and the membrane retained the biomass at a high concentration, so a settling tank was unnecessary.

Growth and decay Although growth of activated sludge as a single microorganism or a functional bacterial group is usually expressed somewhat by kinetics based on the Monod equation (Gujer and Kappeler, 1992; Gujer et al., 1995; Henze et al., 1987), it is difficult to express the growth kinetics of a bacterial strain in activated sludge because there are too many uncertain ecological factors.

Bacterial growth in the process is minimized in order to obtain flocculation and a highly clarified effluent. Growth and decay of bacteria depend on many factors such as nutrients, toxicants, temperature, predation by protozoa, infection of bacteriophage, and so on (Barcina et al., 1997; Peterson and Ward 1989; Roszak and Colwell, 1987). Dispersed growth may be advantageous for bacteria under certain conditions since a maximum surface area would be available for nutrient transport. On the contrary, it is also known that adsorbed bacterial cells onto particles show different assimilation activities (Bar-Or, 1990; Bright and Fletcher, 1983; Diab and Shilo, 1988) and different cell sizes (Brettar et al., 1994) from suspended cells, and protozoa show size-selective grazing on bacteria (Gonzalez, et al., 1990). During starvation, bacteria become more hydrophobic and adhesive and show a higher spontaneous mutation rate; changes in membrane fatty acids, cell wall amino acids and topology of the chromosomes have been described as well as a general enhanced resistance to heat, oxidative, and osmotic shock (Barcina et al., 1997).

Adsorption and desorption The structure of flocs is very porous and the bacteria inside undergo the same nutrient flow as suspended bacteria. This means that flocs cannot be seen as dense, solid particles on bacteria. Before the start of bioaugmentation, of activated sludge, a good settling sludge should preferably be available because the introduced bacteria should adsorb onto flocs to avoid washout from the process (Hess et al., 1993).

Unfortunately, sorption of bacterial cells onto flocs have hardly been studied (van der

Drift et al., 1977; Zita and Hermansson, 1997). Besides, some bacteria have a floc-forming ability, and the mechanisms of floc formation are very complicated (Farrah and Bitton, 1983; Gabriel.Bossier and Verstaete, 1996). It is also remained unknown what kinds of kinetics models are applicable to bacterial adsorption onto flocs.

2-4. SUMMARY

Effect of operational factors, HRT and SRT, on the survival of introduced bacteria in activated sludge were investigated. From the results, a concept for the modeling the behavior of introduced bacteria in activated sludge was proposed. It was hypothesized that the total population of the bacteria should be given by the sum of two sub-populations: that of ones adsorbed onto sludge flocs (X_f) and that of ones suspended in liquid (X_s). Factors affecting the behavior of the sub-populations were discussed and it was concluded that growth/decay and adsorption/desorption of introduced bacteria should be most complicated and important factors for their survival.

Chapter 3. Adsorption of Introduced Bacterial Cells onto Activated Sludge Flocs

3-1. INTRODUCTION

A simple and economical method of bioaugmentation is to introduce the cells directly into the activated sludge during the start-up period. However, it was suggested in Chapter 1 that bacteria directly introduced into activated sludge can not be adsorbed immediately to flocs, resulting in a considerable number of the cells being washed out in the effluent in the rapid declining phase. In Chapter 2, it was also suggested that the hydraulic retention time affects the behavior of the introduced bacteria especially in the rapid declining phase. Some researchers noted that one of the most important factors for the survival of introduced bacteria is their floc formation ability or adsorption ability onto flocs (Fujita et al., 1994a; Kanagawa and Mikami, 1995; McClure et al., 1989), because the flocculated biomass is recycled in the activated sludge process, while the suspended biomass would leave the process in the effluent through the overflow (Shimizu and Odawara, 1985). Therefore, the bacterial adsorption process onto flocs should be fully elucidated to enable effective establishment of strategies for successful bioaugmentation. In this chapter, the adsorption process of 9 bacterial strains were investigated and a kinetic model describing the adsorption process was developed.

3-1. EXPERIMENTALS

Materials and methods

Bacteria and plasmids Bacteria and plasmids used in this chapter are listed in Table 3.1. The plasmids were introduced into the bacteria to provide selective markers for their specific enumeration against the background of indigenous activated sludge bacteria. Plasmid pBH500 was introduced into *Pseudomonas putida* BH and *E. coli* C600, thereby providing its hosts

Table 3.1. Bacteria and plasmids used in this study

Bacteria/plasmids	Relevant properties	Reference
Bacteria		
A. calcoaceticus AH	Phe$^+$, isolated from phenol-acclimated activated sludge	Hashimoto and Fujita, 1987
Alcaligenes sp. YAJ	Phe$^+$, isolated from phenol-acclimated activated sludge	Fujii et al., 1997
Citrobacter sp. TKF04	Glu$^+$, isolated from a kitchen drain biofilm	-
E. coli C600	leu$^-$, thi$^-$, thr$^-$, Des^r	Appleyard, 1954
F. meningosepticum RIMD0614004		RIMD
G. amarae	Oct$^+$, Nal^r, isolated from scum	Sakai et al., 1982
P. putida BH	Ben$^+$, Phe$^+$, isolated from phenol-acclimated activated sludge	Hashimoto and Fujita, 1987
P. fluorescens ATCC15553		ATCC
S. paucimobilis 551	Sta$^+$, Sm^r, isolated from synthetic-wastewater-acclimated activated sludge	Fujita et al., 1991
Plasmid		
pBH500	Sm^r, *pheB*	Fujita et al., 1993a
pS10-45	Km^r, *pheA*, *pheB*, *pheC*, *pheD*, *pheR*	Takeo et al., 1995
RP4	Ap^r, Km^r, Tc^r	Jacob and Grinter, 1975

Abbreviations: resistance to ampicillin (Ap^r), desoxycolate (Des^r), kanamycin (Km^r), nalidixic acid (Nal^r), streptomycin (Sm^r), and tetracycline (Tc^r); requirements for leucine (leu$^-$), thiamine (thi$^-$), and threonine (thr$^-$); growth on benzoate (Ben$^+$), glucose (Glu$^+$), n-octadecane (Oct$^+$), phenol (Phe$^+$), and starch (Sta$^+$).

resistance to streptomycin (Sm). Plasmid RP4 was introduced into *Acinetobactor calcoaceticus* AH, *Alcaligenes* sp. YAJ, *Flavobacterium meningosepticum* RIMD0614004, and *P. fluorescens* ATCC15553, which gives its hosts resistance to ampicilin (Ap), kanamycin (Km), and tetracyclin (Tc). Plasmid pS10-45 was introduced into *Citrobacter* sp. TKF04 and *Sphingomonas paucimobilis* 551, which gives its hosts resistance to Km.

Media Bacteria introduced into the activated sludge were enumerated using plate count techniques with selective media. *Citrobacter* sp. TKF04 (pS10-45) was enumerated using a basal salt medium (Fujita et al., 1995) containing 10 g/*l* of glucose as the sole carbon source with 50 mg/*l* of Km and 50 mg/*l* of cycloheximide, *S. paucimobilis* 551 (pS10-45) using LB medium (Sambrook et al., 1989) with 50 mg/*l* of Km and 50 mg/*l* of Sm, and *G. amarae* using OD medium (Fujita et al., 1994b). *A. calcoaceticus* AH (RP4) and *Alcaligenes* sp. YAJ (RP4)

were enumerated using the basal salt medium containing 500 mg/*l* of phenol as the sole carbon source with 50 mg/*l* of Km and 50 mg/*l* of Ap, *E. coli* C600 (pBH500) using deoxycolate agar (Eiken Chemical, Tokyo) with 50 mg/*l* of Sm (Fujita et al., 1994a), *F. meningosepticum* RIMD0614004 (RP4) and *P. fluorescens* ATCC15553 (RP4) using LB medium with 50 mg/*l* of Ap and 50 mg/*l* of Km, and *P. putida* BH (pBH500) using a basal salt medium containing 500 mg/l of benzoate as the sole carbon source with 50 mg/*l* of Sm. Heterotrophic bacteria in activated sludge were enumerated using CGY medium (Pike et al., 1972). The synthetic wastewater used in the cultivation of a laboratory-activated sludge unit, determination of growth rates, and the floc formation tests of the bacterial strains contains meat extract, 200 mg; peptone, 300 mg; NaCl, 15 mg; KCl, 7.0 mg; $CaCl_2$, 7.0 mg; $MgSO_4$, 5.0 mg; $NaHCO_3$, 105 mg; and 1 liter of tap water (pH 7.2).

Measurements of specific growth rates and floc formation tests The bacterial strains were cultivated on a rotary shaker (100 rpm, 28°C) until the late-log or stationary phase. Floc formation ability was evaluated by the index of flocculation (IF) (Unz and Dondero, 1967) defined as follows: $100 \times (A_t - A_s) / A_t$. A_t indicates the total biomass concentration in synthetic wastewater measured as optical density at 600 nm using a spectrophotometer (UV1200, Shimadzu, Kyoto), and A_s is the biomass concentration of the supernatant after 30-min settling. A large IF value means relatively higher floc formation and/or good settling characteristics of the culture. Specific growth rates were evaluated from the slopes of A_t values in the log phase.

Flocculation activity tests Kaolin clay (Wako Chemical, Osaka) was chosen as the test standard material for measurement of flocculation activity of the bacterial strains. One ml of the culture broth composed of synthetic wastewater (A_{500} = 0.8) and 4 ml of kaoline clay (5 g/*l*) were mixed in a test tube using a vortex mixer for 60 s, and the mixture were allowed to stand for 5 min. By measuring the decrease in turbidity of the upper phase, the degree of flocculation could be determined at 550 nm with the spectrophotometer. Flocculation activity was evaluated as follows: $100 \times [(A_{550}$ of the reference) - (A_{550} of the sample)]/ (A_{550} of the reference) (Kurane et al., 1986). Here, the reference experiments were performed with water instead of the culture broth.

Hydrophobicity tests Hydrophobicity of the bacterial strains was determined by

measuring bacterial adherence to hydrocarbons (BATH) (Rosenberg et al., 1980). Bacteria grown in synthetic wastewater were washed and resuspended in 5 mg/*l* sodium tripolyphosphate buffer. Four ml of washed cells and 1.0 ml of *n*-octane were mixed in a test tube with a vortex mixer for 60 s. The mixtures was allowed to settle for 30 min, and A_{400} of the aqueous phase was measured with a spectrophotometer. The percentage of adherence of *n*-octane adherence is expressed as follows: $100 \times [$ (A_{400} of *n*-octane-free bacterial suspension) - (A_{400} of the aqueous layer) / (A_{400} of *n*-octane-free bacterial suspension).

Bacterial adsorption tests The bacterial strains were grown in LB broth on a rotary shaker (100 rpm, 28°C) to the mid-log phase, harvested by centrifugation (10,000 × g, 10min, 4°C) and washed with 5 mg/*l* sodium tripolyphosphate buffer. The activated sludge flocs were sampled from a laboratory-activated sludge unit acclimated to synthetic wastewater for more than 5 years. The sludge flocs were washed twice with the basal salt medium to remove dissolved organic carbon in liquid phase, and suspended in the basal salt medium to give a MLSS concentration of approximately 2000 mg/*l* (SV_{30} = 20%). Phase contrast microscopic observation of the washed sludge flocs revealed that the presence of a few large protozoa. The bacterial cells were mixed with 100 ml of the sludge flocs in 300-ml Erlenmeyer flasks rotated on a rotary shaker at 100rpm at 28°C for a given time period. For bacterial counts in the mixed liquor, a 5 ml sample was subjected to sonnicated (UD201,TOMY, Tokyo) for 2.5 min to disperse the bacterial cells and sludge flocs. An aliquot of 2 ml from the same sample was obtained and allowed to settle for 30 min in a test tube and bacterial concentrations was also taken from the resultant supernatant.

Experimental results First, the characteristics of the 9 bacterial strains which appeared to be related to the adsorption process were investigated (Table 3.2). The results were expressed as the mean value of the results of 2 or 3 replicates, and the relative errors of the measurements were within 5%. Among the 9 strains, *Citrobacter* sp. TKF04, *G. amarae*, and *S. paucimobilis* 551 (pS10-45) shared similar properties; high floc formation ability, relatively low specific growth rates, and low hydrophobicity of the cells. These 3 strains were considered to be floc-forming bacteria in this study. Except for *Citrobacter* sp. TKF04, the other bacterial strains did not show a significantly high flocculation activity against kaolin clay. *Citrobacter* sp. TKF04 was originally isolated as a bioflocculant-producing bacterium and a high flocculation

Table 3.2. Characteristics of bacterial strains

Bacterial strains	μ_m (h^{-1}) [a]	Floc formation ability (%) [b]	Flocculation ability (%) [c]	Hydro-phobicity (%) [d]	Lag time (hr)	Freundlich parameters			
						K (-) [e]	m (-) [e]	A (h^{-1}) [f]	D (h^{-1}) [g]
A. calcoaceticus AH (RP4)	4.6×10^{-1}	3	1.0	55	6.4	36	1.1	6.5×10^{-2}	1.8×10^{-3}
Alcaligenes sp. YAJ (RP4)	5.3×10^{-1}	3	1.7	23	6.0	62	0.81	5.4×10^{-1}	8.7×10^{-3}
Citrobacter sp. TKF04 (pS10-45)	3.8×10^{-2}	12	1.0	0	6.0	4.5	1.4	1.2×10^{-3}	2.6×10^{-4}
E. coli C600 (pBH500)	5.0×10^{-1}	3	8.9	2.6	5.3	4.8	1.2	1.5×10^{-3}	3.1×10^{-4}
F. meningosepticum RIMD0614004 (RP4)	6.4×10^{-1}	3	8.7	31	12	18	0.99	6.7×10^{-1}	3.7×10^{-4}
G. amarae	1.1×10^{-1}	9.3	11	0	3.1	20	1.1	2.2×10^{-2}	1.1×10^{-3}
P. fluorescens ATCC15553 (RP4)	4.4×10^{-1}	3	17	34	11	20	1.0	4.1×10^{-1}	7.5×10^{-3}
P. putida BH (pBH500)	5.2×10^{-1}	3	4.0	28	6.5	17	1.1	6.4×10^{-2}	2.1×10^{-2}
S. paucimobilis 551 (pS10-45)	8.9×10^{-2}	40	17	2.8	5.0	0.066	1.7	2.9×10^{-5}	4.4×10^{-4}

[a] Specific growth rate in synthetic wastewater. [b] IF value. [c] Kaolin removal (%) by culture broth. [d] Retained cells in BATH assay. [e] Freundlich equilibrium parameters. [f] Specific adsorption rate. [g] Specific desorption rate.

ability of more than 95% could be obtained when it was cultivated in the basal salt medium containing 10 g/*l* of acetate (data not shown). *G. amarae* is known as a scum-forming microorganism (Sakai et al., 1982), however, abnormal foam formation by the strain was not observed when it was grown in synthetic wastewater in this study.

Typical time courses of bacterial adsorption onto sludge flocs are shown in Figs. 3.1 and 3.2. Each experiment was conducted more than twice. The population of heterotrophic bacteria in activated sludge without any augmentation were remained stable at 1.1×10^9 CFU/ml in mixed liquor and 8.2×10^6 CFU/ml in the supernatant (data not shown). The typical time course shows a triphasic process, consisting of lag, rapid adsorption, and stationary phases. The durations of the three phases depended on the bacterial strain. For example, the lag phase in the adsorption of *E. coli* C600 (pBH500) was observed for 5-7 h, and viable counts in the activated sludge mixture showed smaller values than that in the supernatant (Fig.3.2). A reason for the larger value of the introduced bacterial cells in the supernatant could be that the introduced cells could not immediately elucidate into the sludge volume (SV_{30} = *c.a.* 20%). In the rapid adsorption phase, the cells in the supernatant decreased drastically from 1.4×10^8 to 1.7×10^6 CFU/ml followed by the stationary phase after 15 h. Total population of the introduced bacterial cells were relatively stable for over 24 h, indicating neither drastic growth nor decay of

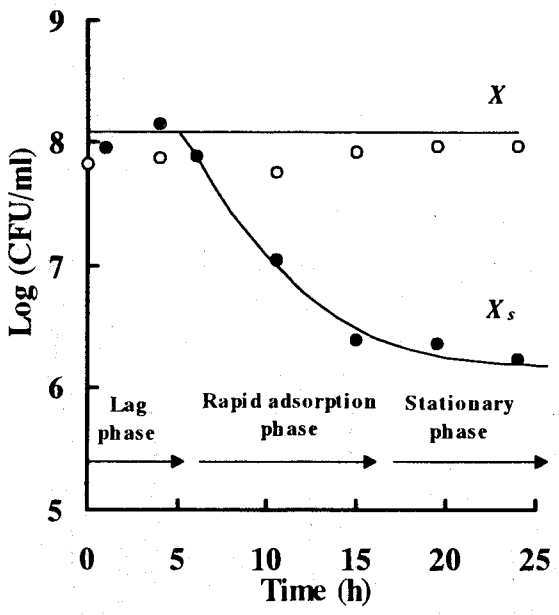

Fig. 3.1. Triphasic adsorption of *E. coli* C600 (pBH500) onto activated sludge flocs (MLSS 2000 mg/*l*, 28°C). Cells in mixed liquor (○) and supernatant (●) are shown. Regression lines of X_s and X are also shown.

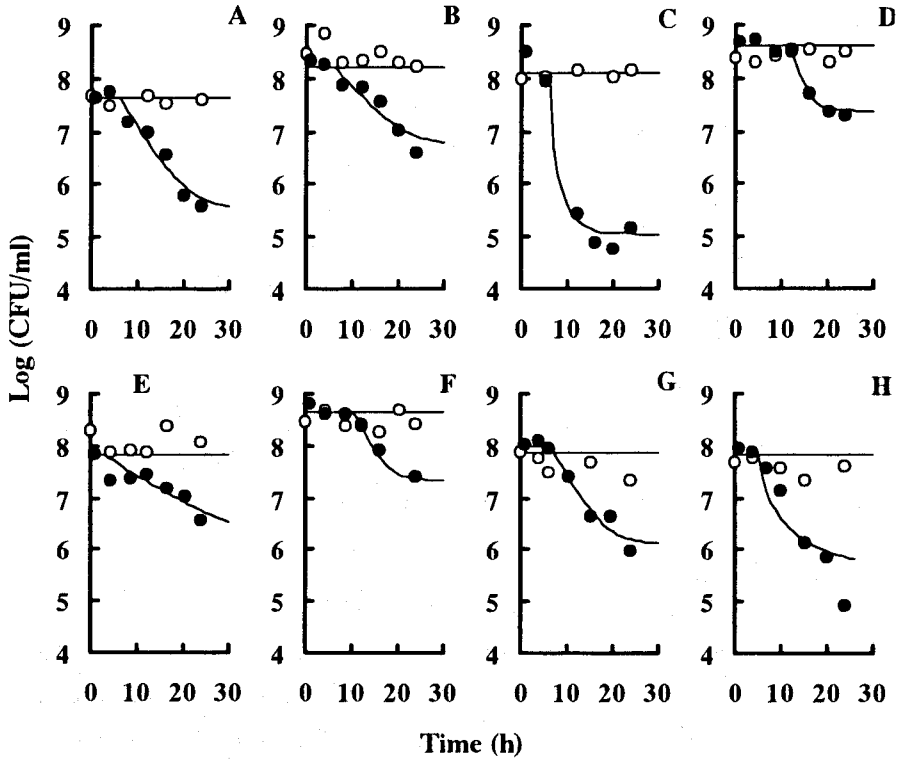

Fig. 3.2. Time courses of bacterial adsorption onto activated sludge flocs (MLSS 2000 mg/*l*, 28°C). (A) *A. calcoaceticus* AH (RP4), (B) *Alcaligenes* sp. YAJ (RP4), (C) *Citrobacter* sp. TKF04 (pS10-45), (D) *F. meningosepticum* RIMD0614004 (RP4), (E) *G. amarae*, (F) *P. fluorescens* ATCC1553 (RP4), (G) *P. putida* BH (pBH500), and (H) *S. paucimobilis* 551 (pS10-45). Cells in mixed liquor (○) and supernatant (●) are shown.

E. coli C600 (pBH500) in activated sludge during the experimental periods. Changes in the introduced bacterial population in the supernatant, therefore, indicate the adsorption of the cells onto sludge flocs.

Data obtained at 24 h after introduction at different cell concentrations of *E. coli* C600 (pBH500) and of other strains followed the Freundlich isotherm (Figs. 3.3 and 3.4). The detection limit of each bacterial strain can be observed in each figure.

3-3. THEORETICAL CONSIDERATIONS

Adsorbed bacterial cells onto sludge flocs can be defined as

$$X_f = X - X_s \quad (3.1)$$

where X_s : sub-population density of the introduced bacterium in the supernatant after 30-min

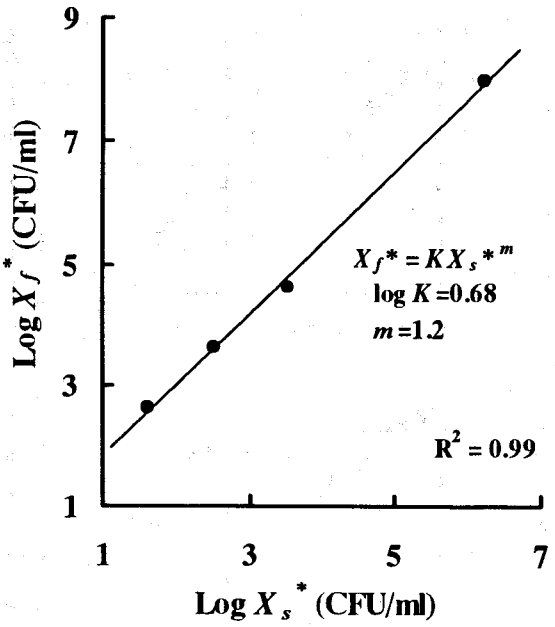

Fig. 3.3. Equilibrium of *E. coli* C600 (pBH500) in activated sludge at 24 hours after introduction (MLSS 2000 mg/*l*, 28°C). The data were fitted to the equation $X_f^* = KX_s^{*m}$, where X_f^* is the adsorbed cells and X_s^* is the aqueous equilibrium cell concentration. The slope and the intercept of the regression line correspond to m and logarithmic K values, respectively.

settling (CFU/ml), X : total population density of the introduced bacterium in the mixed liquor (CFU/ml), and X_f : sub-population density of the introduced bacterium adsorbed onto sludge flocs (CFU/ml).

A simple first-order mass transfer model (Travis and Etnier, 1981; Vilker, 1980), which is frequently used to describe the sorption-desorption relationship between a solute and soil matrix, was thought to describe the bacterial adsorption process. This relationship assumes that the adsorption is nonlinear, instantaneous, and reversible.

$$\frac{dX_s}{dt} = -AX_s^m + DX_f \qquad (3.2)$$

where A : specific adsorption rate of the introduced bacterium (h^{-1}) which accounts for the diffusive transport of the cells through a quiescent liquid layer surrounding each sludge floc, D : specific desorption rate of the introduced bacteria from sludge flocs (h^{-1}), and m : an empirical constant.

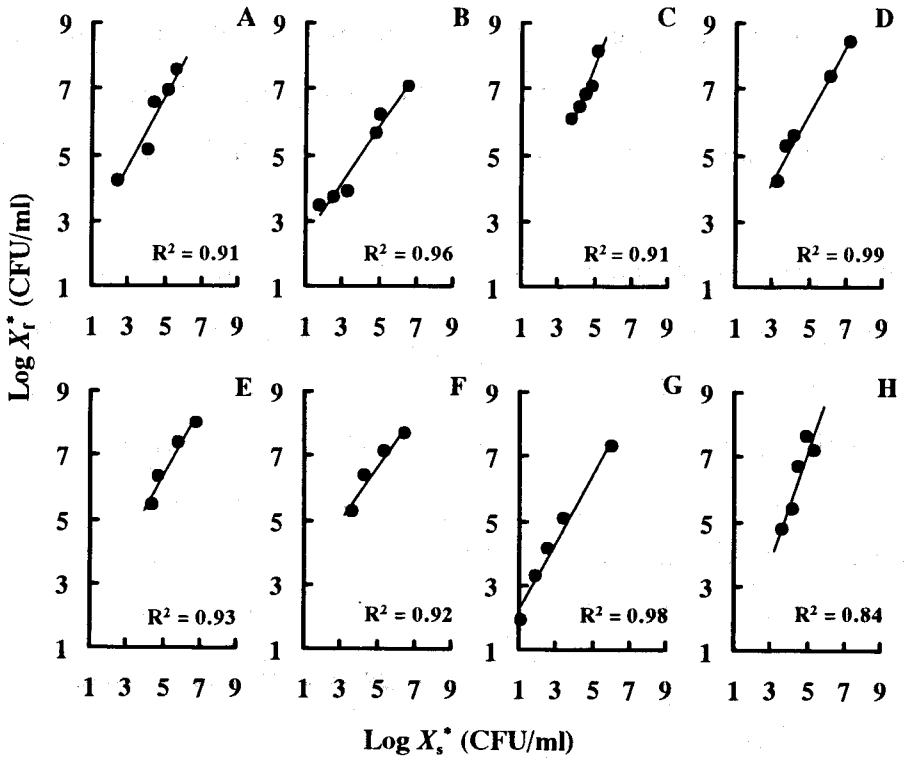

Fig. 3.4. Sorption isotherms for bacteria onto activated sludge flocs at 24 hours after introduction (MLSS 2000 mg/*l*, 28°C). (A) *A. calcoaceticus* AH (RP4), (B) *Alcaligenes* sp. YAJ (RP4), (C) *Citrobacter* sp. TKF04 (pS10-45), (D) *F. meningosepticum* RIMD0614004 (RP4), (E) *G. amarae*, (F) *P. fluorescens* ATCC1553 (RP4), (G) *P. putida* BH (pBH500), and (H) *S. paucimobilis* 551 (pS10-45).

In a stationary phase, equation 3.2 is rewritten as follows:

$$X_f^* = K\,(X_s^*)^{\,m} \qquad (3.3)$$

where X_s^* : X_s in the stationary phase (CFU/ml), X^* : X in the stationary phase (CFU/ml), X_f^* : X_f in the stationary phase (CFU/ml), and K ($=A/D$): an empirical constant. Equation 3 corresponds to the Freundlich isotherm and the flexibility of the two constants, K and m, allows easy curve fitting. Larger values of the two constants indicate a higher adsorption property, and the m value effects are more sensitive than that of the K value.

The isotherms shown in Figs. 3.3 and 3.4 are plotted in log-log scale and the slopes and the intercepts of the regression lines indicate the m values and log of the K values (Table 3.2). The equations for the best lines gave positive K values in all cases, implying that the isotherms

were not linear over the whole concentration range. Two of the floc-forming bacteria, *Citrobacter* sp. TKF04 and *S. paucimobilis* 551 (pS10-45), showed relatively small K and large m values. The regression lines indicate that if X_s is below 10^3 CFU/ml, *A. calcoaceticus* AH (RP4) adsorbs onto sludge flocs at the highest ratio among the 9 strains, while *Citrobacter* TKF04 (pS10-45) and *S. paucimobilis* 551 (pS10-45) adsorb at the highest ratio of $10^3<X_s<10^6$ and $10^6<X_s$, respectively.

After the estimation of K and m for each bacterial adsorption onto flocs, A and D were optimized by fitting the solutions to the data using least-squares analyses by FORTRAN compilation (Figs. 3.1 and 3.2). Because of the above-stated reasons, constant X values and lag times were also optimized with A and D ($=A/K$) values by the numerical solution (Table 3.2). The regression lines calculated with the estimated parameters showed good fittings to the data, indicating a nonlinear adsorption reaction of the cells onto sludge flocs and a linear desorption reaction from sludge flocs. Relatively low sorption values of A and D were commonly obtained for the two floc-forming bacteria, *Citrobacter* TKF04 (pS10-45) and *S. paucimobilis* 551 (pS10-45).

3-4. DISCUSSION

Adsorption of introduced bacteria onto sludge flocs was studied to develop a sub-model for describing the behavior of introduced bacteria in activated sludge. A novel finding is that the adsorption process of bacterial cells is generally triphasic consisting of lag, rapid adsorption, and stationary phases. To the best of our knowledge, only one previous report has described the dynamic process of bacterial adsorption onto activated sludge flocs; however, no lag phase was observed (van der Drift et al., 1977). This previous study was performed using only *E. coli* V2086 cells for the adsorption tests. On the other hand, 9 different kinds of bacterial cells were used in our present study; therefore, the observed triphasic process, *i.e.*, presence of the lag phase, of the bacterial adsorption onto sludge flocs seems to be a generalized phenomena in real activated sludge processes. The presence of the lag phase indicates that a certain period is necessary before the bacterial cells introduced into the activated sludge start to be adsorbed onto the flocs. It suggests that temporarily stopping of influent feeding during the activated sludge process may be required for a certain period after the introduction of bacteria for effective bioaugmentation. Within the scope of this study, the reason(s) for the presence of the lag phase was not clarified. There were many differences between the bacterial adsorption test performed in our study and that in the previous study; *e.g.* properties of activated sludge and

bacterial strains used, compositions and pH of the test media, shearing force loaded during the tests, and temperature. The bacterial adsorption process should be affected by many factors including the above-mentioned ones, and further intensive studies are necessary to elucidate the reasons for the presence of the lag time.

The data obtained during the stationary phase were well fitted by a reversible and nonlinear kinetic model which corresponds to the Freundlich isotherm. The above previous study reported that the adsorption of *E. coli* V2086 onto sludge flocs obeyed the Langmuir isotherm (van der Drift et al., 1977). The Freundlich isotherm observed for the bacterial sorption equilibrium in this study can be regarded as a modified form of the Langmuir isotherm (Travis and Etnier, 1981). However, it is not possible to compare the estimated equilibrium parameters in this study with those of the previous report since the report showed no quantitative data and graphs or parameters on the isotherm, thus, no kinetic model has ever been applied to the process of bacterial adsorption onto sludge flocs. A limitation of the Freundlich isotherm is that it does not imply a maximum quantity of adsorption as in linear isotherm models. However, the reversible and nonlinear kinetic model proposed based on the Freundlich isotherm could express the adsorption process to a certain degree. The proposed model and estimated parameters in this study will be helpful for further model development of bioaugmented activated sludge treatment although further improvement of the model is required to simulate the process in the lag phase. The two-site kinetic model which is much more complicated but can describe the sorption process of bacterial cells to soil particles may be an alternative model (Lindqvist and Bengtsson, 1991).

The adsorption tests were carried out using a variety of bacterial strains; however, there was no significant relationship between the adsorption parameters and their characteristics except for their floc-forming ability. The three floc-forming bacteria, *Citrobacter* sp. TKF04, *G. amarae*, and *S. paucimobilis* 551 (pS10-45), showed common properties: low specific growth rates and hydrophobicity. Floc-forming bacteria seem useful for bioaugmentation to activated sludge because they are difficult to wash out, thus giving them a competitive advantage over suspended bacteria. The two bacterial strains, *Citrobacter* sp. TKF04 and *S. paucimobilis* 551 (pS10-45), which seem to be typical floc-forming bacteria have small K, A, D and large m values. The small K and large m values mean that high adsorption ratios can be obtained when these bacterial strains are introduced at high densities. The low specific sorption rates mean that the bacterial adsorption process requires a long rapid adsorption phase. Therefore, high inoculum densities and long hydraulic retention times are required for effective

bioaugmentation of floc-forming bacteria.

A previous study reported that increased cell surface hydrophobicity of *E. coli* strains correlate well with increased adsorption onto sludge flocs (Zita and Hermansson, 1997). On the contrary, many researchers stated that the scum formation in activated sludge processes are due to acinetomycetes (*e.g. G. amarae* and *Rhodococcus* spp.) which possess strong hydrophobic properties, *i. e.*, the hydrophobic properties of acinetomycetes seem to have a negative effect on their adsorption onto sludge flocs (Sakai et al., 1982). This contradiction suggests that the relationship between bacterial hydrophobicity and adsorption onto sludge flocs may depend on the bacterial species. It may be a possible reason why no significant relationship between hydrophobicity of bacterial cells and their adsorption was observed in this study. Another possible reason is that other properties of bacterial strains had greater effects on their adsorption onto sludge flocs than their hydrophobicity. For example, it was reported that the presence of fimbriae, production of extracellular polymers, and an increase in positive surface charge increased bacterial adsorption ability onto sludge flocs (Zita and Hermansson, 1997). From a practical view point, the genes responsible for the production of fimbriae and bioflocculants may be possible components for constructing useful recombinant bacterial strains with high survival ability in activated sludge. It is also likely that several unknown physiological or morphological properties of the bacteria - interaction with sludge flocs - determine their adsorption capabilities. Further work is required to define the specific characteristics, in addition to floc formation ability, that allow introduced bacteria to be efficiently adsorbed onto sludge flocs.

3-5. SUMMARY

The adsorption of bacterial cells of 9 species onto activated sludge flocs were investigated and a kinetic model of the adsorption process was developed. The typical time course of bacterial adsorption, which is a triphasic process, consisted of lag, rapid adsorption, and stationary phases. The equilibrium of the cells in the stationary phase obeyed the Freundlich isotherm. The reversible and nonlinear model could express the process to a certain degree and the Freundlich parameters and specific sorption rates were estimated for each bacterial strains.

Chapter 4. Ecological Factors Affecting the Behavior of Introduced Bacteria in Activated Sludge

4-1. INTRODUCTION

It was suggested in Chapters 1 and 2 that introduced bacteria in activated sludge encountered strong selective pressures which may include starvation, protozoan predation, competition with indigenous bacteria. It was also suggested that the sludge retention time affects the growth/decay of introduced bacteria especially in the slow declining phase. Several abiotic factors affecting the growth/decay of bacteria in natural ecosystems have been suggested such as pH, temperature (Bolton et al., 1991), radiation (Barcina et al., 1996), nutrients (Sinclair and Alexander, 1989; Wang and Bakken, 1998), toxins (Klein and Alexander, 1986), and osmotic pressure (Mezurioui et al., 1995). On the other hand, influential biotic factors are microbial interactions such as competition with indigenous bacteria and predation by protozoa (Amy et al., 1989; Gurijara and Alexander, 1990; Iwasaki et al., 1993; Recorbet et al., 1992; Sinclair and Alexander, 1984; Tang and Alexander, 1987). However, not much useful knowledge has been obtained to date especially on the biotic factors, and further studies are required to obtain more generalized knowledge.

In this chapter, first, effect of starvation, protozoan grazing, and competition with indigenous bacteria on the introduced bacteria, *Pseudomonas putida* BH (pBH500) and *Escherichia coli* K-12 (pBH500), were studied using activated sludge microcosms. Second, simulation studies on the interaction between introduced and indigenous bacterial strains, were carried out. The knowledge of such ecological factors should facilitate the assessment of the likelihood that introduced bacteria in activated sludge will survive and help to select bacteria whose period of survival may be either long or short as desired for particular functions or uses.

4-2. EXPERIMENTALS

Materials and methods

Bacterial strains and culture media *P. putida* BH (pBH500) and *E. coli* K-12 (pBH500) were introduced into activated sludge microcosms. Counts of *E. coli* K-12 (pBH500) and *P. putida* BH (pBH500) introduced into microcosms were determined on Desoxycolate agar (Eiken Chemical, Tokyo) and a basal salt medium (Fujita et al., 1994a) containing 500 mg/l benzoate, respectively. Both media were supplemented with 50 mg/*l* Sm. The combination of these selective media and spraying colonies with catechol enabled specific detection of the introduced bacteria in all the microcosms used with a detectable limit of 10 CFU/ml. For indigenous heterotrophic bacteria in activated sludge, CGY medium (Pike et al., 1972) was used. For routine maintenance of the bacterial strains, LB broth (Sambrook et al., 1989) was used.

Activated sludge microcosms Activated sludge microcosms consisted of 300-ml Erlenmeyer flasks containing a 100 ml sample obtained from a municipal sewage treatment plant (Osaka Prefecture) in July, 1996. To individually evaluate the effects of predation by protozoa, interactions with indigenous bacteria, and starvation, intact, protozoa-free, and sterilized microcosms were prepared, and the survival courses of the introduced bacteria in this series of microcosms and in a sterile buffer were examined. The freshly obtained activated sludge was washed twice with 5 mg/*l* sterile sodium tripolyphosphate solution to remove dissolved organic carbon in the liquid phase, re-suspended in the original volume of a basal salt medium, and used as the intact microcosm (MLSS 1400 mg/*l*, DOC 10 mg/*l*, pH 7.2). An eucaryotic inhibitor, cycloheximide (2.0 g/*l*), was added to the washed activated sludge for preparing the protozoa-free microcosm. The sterilized microcosm was obtained by autoclaving (120℃, 20 min) the washed activated sludge. Sludge wastage and effluent discharge from the microcosms were not carried out without sampling of mixed liquor for enumeration of microorganisms.

Survival study *P. putida* BH (pBH500) and *E. coli* K-12 (pBH500) were grown overnight to the mid-log phase in LB broth supplemented with Sm (50 mg/*l*). Cells were harvested by centrifugation at 15000×g at 4℃ for 10 min, washed twice with sodium tripolyphosphate solution, and introduced into the microcosms at appropriate densities. The

activated sludge microcosms were incubated at 28℃ on a rotary shaker operating at 100 rpm. The numbers of the surviving bacteria and behavior of indigenous microorganisms were monitored periodically. Bacteria in the microcosms were counted by plate count techniques using the above-mentioned media. Samples from the activated sludge microcosms were treated with a sonicator (UD201: TOMY, Tokyo) to disperse the sludge flocs (Fujita et al., 1994a) for bacterial enumeration. All the plates were incubated at 28℃ for 2 d for counting *P. putida* BH (pBH500) and *E. coli* K-12 (pBH500), and for 7-10 d for counting indigenous heterotrophic bacteria. Protozoa except small types such as nano-flagellates in the activated sludge microcosms were measured with a phase contrast microscope at ×100 or ×200 magnification. As control experiments, the survival of the introduced bacteria in sterile buffers containing no carbon source was investigated for evaluating the effect of starvation. The buffer used for this purpose was a basal salt medium.

Screening of antagonistic bacteria Bacteria which inhibit the growth of the introduced bacteria, *P. putida* BH (pBH500) and *E. coli* K-12 (pBH500), were defined as antagonistic bacteria. About 200 colonies of indigenous heterotrophic bacteria were isolated from the sample (the intact microcosm) by using CGY medium and grown at 28℃ for 3-5 d. A small amount of cell paste was scraped off and deposited onto the surface of the same medium plates seeded with *P. putida* BH (pBH500) or *E. coli* K-12 (pBH500), and the plates were incubated at 28℃ for 2-3 d. A clear zone of inhibition formed around the bacteria from the paste indicated the antagonistic activity against *P. putida* BH (pBH500) or *E. coli* K-12 (pBH500).

Experimental results

Survival of introduced bacteria in activated sludge microcosms Results of the survival studies of the introduced bacteria in the activated sludge microcosms are shown in Figs. 4.1 and 4.2. *P. putida* BH (pBH500) and *E. coli* K-12 (pBH500) were introduced into the microcosms at densities of 2.9×10^7 and 5.0×10^8 CFU/ml, respectively. Large viable protozoa were not observed in the protozoa-free microcosms, and no indigenous bacteria were recovered from the sterilized microcosms. The intact microcosm introduced with *P. putida* BH (pBH500) was lost due to some trouble on day 6. Both types of introduced bacteria declined in population by 2 to 3 orders of magnitude over 5 or 7 d in the intact and protozoa-

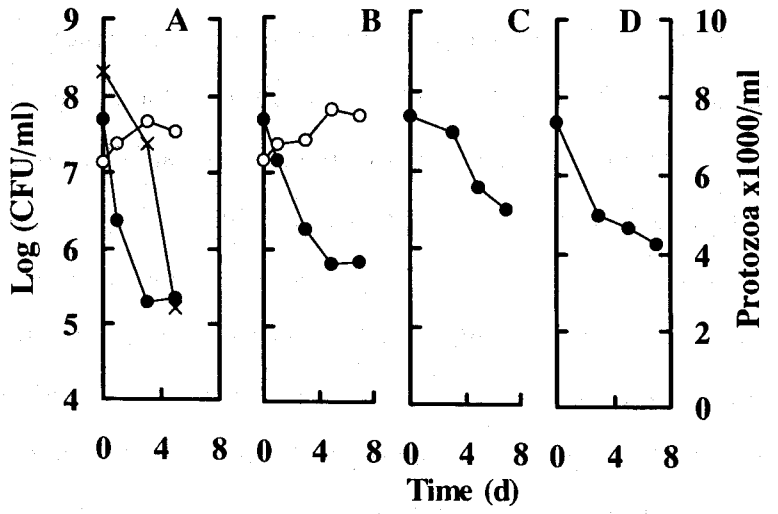

FIG. 4.1. Survival of *P. putida* BH (pBH500) in the activated sludge microcosms. Populations of *P. putida* BH (pBH500) (●), indigenous heterotrophic bacteria (○), and protozoa (×) in the intact microcosm (A), protozoa-free microcosm (B), sterilized microcosm (C), and sterile basal salt medium (D) are shown.

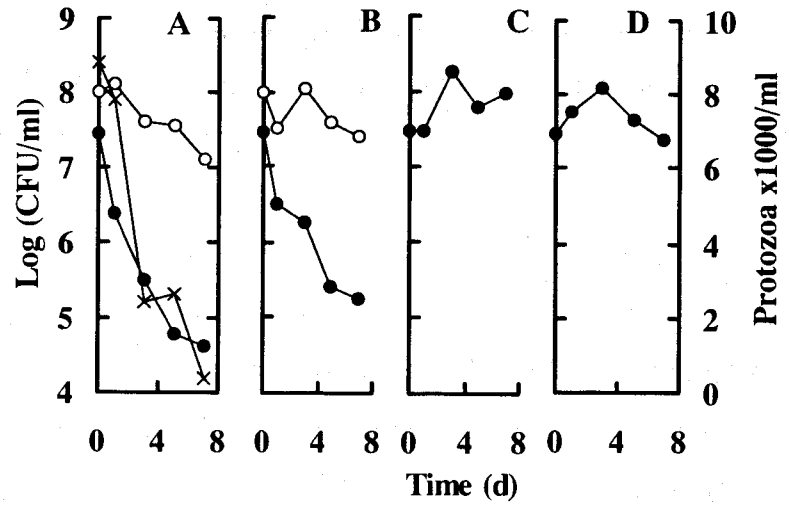

FIG. 4.2. Survival of *E. coli* K-12 (pBH500) in the activated sludge microcosms. Populations of *E. coli* K-12 (pBH500) (●), indigenous heterotrophic bacteria (○), and protozoa (×) in the intact microcosm (A), protozoa-free microcosm (B), sterilized microcosm (C), and sterile basal salt medium (D) are shown.

free microcosms, and they survived at little higher levels in the protozoa-free microcosm than in the intact microcosm. In the sterilized microcosm and in the sterile buffer, the *E. coli* K-12 (pBH500) population was maintained at stable levels over 7 d, while that of *P. putida* BH (pBH500) gradually decreased. Protozoa decreased drastically in the intact microcosms, and the effect of protozoan predation might therefore be underestimated from that in natural conditions. A decrease in the protozoan population was also observed in the intact microcosms without inoculation of the introduced bacteria (data not shown). However, the surviving protozoa such as *Zoothamnium* and *Aspidisca* were frequently observed to show grazing activity.

The survival in the sterile buffers should represent a basic decay property of the tested bacteria as affected by starvation in the absence of specific abiotic selective factors, while the pattern in the intact microcosms as that affected by all the abiotic and biotic factors. Addition of cycloheximide, which was performed in preparing the protozoa-free microcosms, was thought to considerably reduce the effect of protozoan grazing, though such a treatment seems to be unable to completely kill protozoa in the microcosms. Autoclaving performed in preparing the sterilized microcosms should have eliminated all the biotic factors. Thus, the differences between the survival patterns in the intact microcosm and in the protozoa-free microcosm, between those in the protozoa-free microcosm and in the sterilized microcosm, and between those in the sterilized microcosm and in the sterile buffer should reflect to a certain degree the effects of the protozoa, indigenous bacteria, and starvation along with abiotic factors, respectively.

From the data, it was indicated that the interaction with indigenous bacteria was the most influential factor causing the decline of the introduced bacterial populations, and it seemed that this disadvantageous interaction between indigenous and introduced bacteria leading to lethal effects took the form of the antagonism or amensalism rather than competition for nutrients. Some researchers reported that protozoan predation is to be a possible important factor affecting the survival of bacteria introduced into fresh water (Amy et al., 1989; Gurijara and Alexander, 1990; Iwasaki et al., 1993), sewage (Sinclair and Alexander, 1984), and soil (Tang and Alexander, 1987), and its effect on the survival of introduced bacteria was certainly observed in this study. However, it seemed that protozoan predation was much less influential than the existence of indigenous bacteria in activated sludge. Although the effect of protozoan predation in activated sludge might be underestimated owing to the decrease in their population, the survival tests here seem to be able to reflect the

predatory effects to a certain degree because vigorous grazing activities of surviving protozoa were often observed during the test period. It was also reported that protozoan predation was not an important lethal factor working against introduced bacteria in a model activated sludge process (McClure et al., 1989) and soil (Zaidi et al., 1989).

Screening of indigenous bacteria with antagonistic activity To provide evidence for the presence of antagonism between indigenous and introduced bacteria, bacterial strains having antagonistic activities against *P. putida* BH (pBH500) or *E. coli* K-12 (pBH500) were screened from environmental samples used for the survival studies. In total 208 indigenous bacterial strains were isolated from the microcosm, and there were 2 strains identified as antagonistic to *P. putida* BH (pBH500) and 5 to *E. coli* K-12 (pBH500). There are no bacterial strain could inhibit the growth of both *P. putida* BH (pBH500) and *E. coli* K-12 (pBH500), suggesting that an antibacterial factor produced by an antagonistic bacterial strain has lethal effects on a limited number of bacterial species or strains. Interestingly, all the antagonistic bacteria lost their antagonistic activity during the two or three transfers to new growth media, indicating that they need some specific conditions to exhibit antagonistic activities, such as trace substances present in their native microbial ecosystems.

To date, sea water has been known to contain substances with antagonistic activity, and it has been suggested that such substances are produced by marine algae or bacteria (Dopazo et al., 1988; Gauthier and Fratas, 1976; Lemos et al., 1985; Rosenfeld and ZoBell, 1947). It was reported that about 5% of bacteria isolated from sea water exhibited inhibitory activity against the growth of either *Vibrio parahaemolyticus* or *Staphylococcus aureus* (Nair and Simidu, 1987), and about 17% of bacteria isolated from intertidal seaweeds exhibited antagonistic activity against *Staphylococcus aureus* (Lemos et al., 1985). Some antagonistic substances produced by marine bacteria were low molecular weight compounds, thermolabile, anionic, and not affected by proteolytic enzymes (Lemos et al., 1985). Klein and Alexander (1986) also reported the existence of antagonistic substances, which were low molecular weight compounds, thermolabile, and cationic, in lake water.

Timmis (1997) improved the survival of introduced bacteria in soil by pre-adaptation of the strains on a minimal medium with soil extract. Pre-adaptation of the bacterial strains to the activated sludge conditions, before introduction into activated sludge, could possibly help to solve the survival problems caused by the inhibitory stress.

4-4. THEORETICAL CONSIDERATIONS

Indigenous bacteria with antagonistic activity From the experimental results, it was concluded that the antagonistic activities of indigenous bacteria is the most significant factor which influences the ability of introduced bacteria to survive in activated sludge. There have been several reports on the presence of antagonistic relationships among indigenous bacteria; however, no report has clearly asserted that this antagonism is the most lethal factor affecting the survival of introduced bacteria in any microbial ecosystem. For successful bioaugmentation to activated sludge where indigenous antagonistic bacteria exist, it is necessary to understand the ecological role of the antagonism. Here, antagonistic competition of two bacterial strains in a completely mixed reactor was simulated with a numerical technique in 12 cases depending on the disposition of the growth rate curves of the two bacterial strains and on the sensitivity of the bacterial strains against the substance.

Governing equations For simplification, it was assumed here that bacterial strains 1 and 2 compete for a common substrate in an idealized completely mixed reactor (Fig. 4.3). In addition, it was assumed that the production rate of the inhibitory substance was proportional to the growth rate, and neither bacteria consumes the inhibitory substance. The mass balances in the reactor are expressed by following equations:

$$\frac{dX_i}{dt} = (\mu_i - \frac{1}{\theta})X_i \qquad (4.1)$$

$$\frac{dS}{dt} = \frac{S_f - S}{\theta} - \sum \frac{\mu_i X_i}{Y_i} \qquad (4.2)$$

$$\frac{dI}{dt} = -\frac{I}{\theta} + \sum p_i \mu_i X_i \qquad (4.3)$$

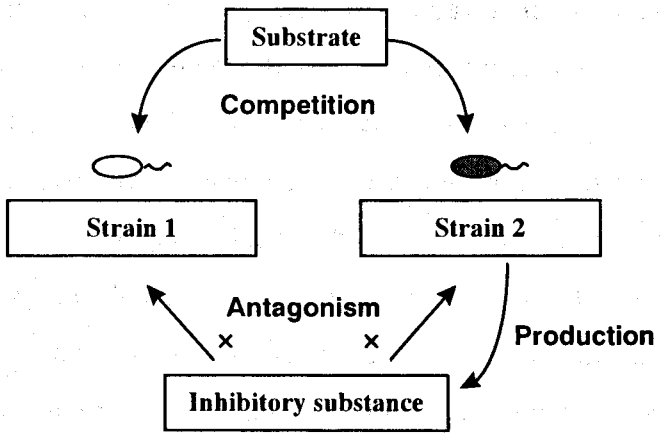

Fig. 4.3. Antagonistic relationship between bacterial strain 1 and bacterial strain 2.

where X, S, and I are bacterial population density, concentration of the substrate, and concentration of the inhibitory substance in the reactor, respectively. S_f and θ are substrate concentration in influent and hydraulic (cell) retention time. μ, Y, and p are specific growth rate, yield constant, and production constant of the inhibitory substance of the i th bacterial strain. The growth of the bacterial strains are expressed by a following equation which is a modified Monod kinetics for metabolite inhibition (Han and Levenspiel, 1988):

$$\mu_i = \frac{\mu_{mi} S}{K_{Si} + S}\left(1 - \frac{I}{K_{Ii}}\right) \qquad (4.4)$$

where μ_m, K_S, and K_I are maximum growth rate, half saturation constant, and inhibition constant for the i th bacterial strain, respectively.

In view of the inhibitory mechanism, there are two types of inhibitory substances; one shows inhibition against the growth (Lemos et al., 1985; Nair and Simidu, 1987; Rosenfeld and ZoBell, 1947) and the other is lethal to bacteria (Jemsen, et al., 1993; Tang and Alexander, 1987). In this model, the parameter K_I indicates the concentration of the inhibitory substance ceases to grow of the bacterial strain. When the concentration of the inhibitory substance is higher than the K_I value, the specific growth rate becomes below zero and indicates decay of the bacteria. The small K_I value means that the microorganism is subject to considerable inhibition.

Parameters Bacterial strains 1 and 2 are assumed as an introduced bacteria and an indigenous bacteria, respectively, *i. e.*, it was assumed that only bacterial strain 2 produces the inhibitory substance. Some inhibitory substances show inhibition against its producer (autoinhibition) (Lemos et al., 1985; Nair and Shimidu, 1987), therefore, three cases, 1-3, were considered as shown in Table 4.1. In case 1, bacterial strain 2 is not affected by the inhibitory substance. In case 2, bacterial strain 2 is as sensitive as bacterial strain 1 to the inhibitory substance. In case3, bacterial strain 2 is more sensitive to the inhibitory substance than bacterial strain 1. Thus, survival of bacteria depends on the disposition of their growth rate curves (Aris and Humphrey, 1977). Therefore, 4 cases of the disposition, A-D, were assumed as shown in Table 4.2. The dispositions of the growth rate curves when their growth are not affected by the inhibitory substance are shown in Fig. 4.2 ($K_{I1} = K_{I2} = \infty$). In case A, bacterial strain 2 grows faster than bacterial strain 1 at any substrate concentration. In case B, bacterial strain 1 grows faster than bacterial strain 2 at any substrate concentration. In case C, bacterial strain 1 grows faster at high substrate concentrations. In case D, bacterial strain 2 grows faster at high substrate concentrations. On the whole, 12 cases, combination of cases 1-3 and cases A-D, were assumed. All simulations were carried out using the 4th order Runge-kutta method provided by the software ISIM for Windows (Snape et al., 1995).

Operational diagrams The nature of solutions of the equations depends on the microbial parameters in each case and on the operational parameters, θ and S_f. This dependence is summarized on an operational diagram, which is a θ^{-1} - S_f plane where various steady-state solution of the model equations exist and are stable with respect to small perturbations. The diagrams in the 12 cases consist of 5 regions, I-V (Fig. 4.4). In region I, both bacterial strains are washed out from the reactor. In regions II, bacterial strain 1 is washed out but bacterial strain 2 survives in the reactor. Conversely, in region III, bacterial strain 2 is washed out but bacterial strain 1 survives in the reactor. In region IV, bacterial strain 1 or bacterial strain 2 is washed out depending on the initial conditions of S, I, X_1, and X_2 (multi-steady-state), indicating a large inoculum density may be effective for bioaugmentation. Interestingly, in region V, the stable steady-state is the coexistence of bacterial strains 1 and 2. Regions IV and V are not observed when the typical Monod equation is applied to simulations of the competition of two bacterial strains for a single substrate (Aris and Humphrey, 1977). Region IV is observed in cases (1-B), (1-D), (2-D), and (3-D), and

Table 4.1. Parameters of the bacterial strains concerning the inhibitory substance in cases 1-3

Case	K_{I1} (mg/l)	K_{I2} (mg/l)	p_1 (-)	p_2 (-)
1	1.0	∞	0.0	0.1
2	1.0	1.0	0.0	0.1
3	1.0	0.5	0.0	0.1

Table 4.2. Parameters of the bacterial strains concerning the disposition of the growth rate curves in cases A-D

Case	μ_{m1} (d^{-1})	K_{S1} (mg/l)	μ_{m2} (d^{-1})	K_{S2} (mg/l)
A	0.5	25	1.0	25
B	1.2	25		
C	2	150		
D	0.7	1.0		

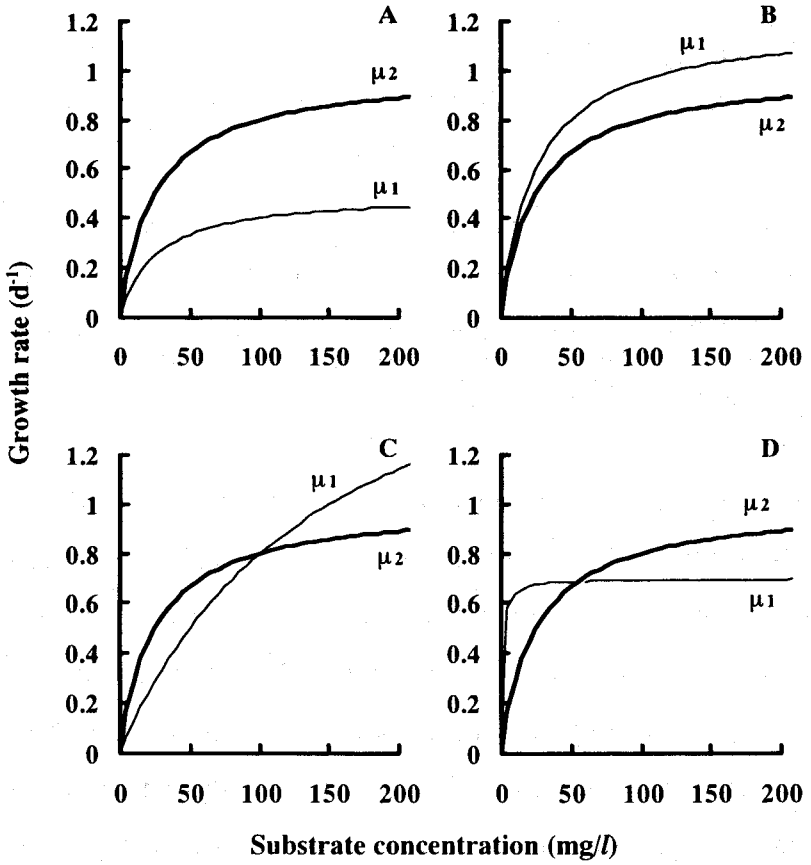

Fig. 4.4. Disposition of growth rate curves of the bacterial strains when their growth is not affected by the inhibitory substance.

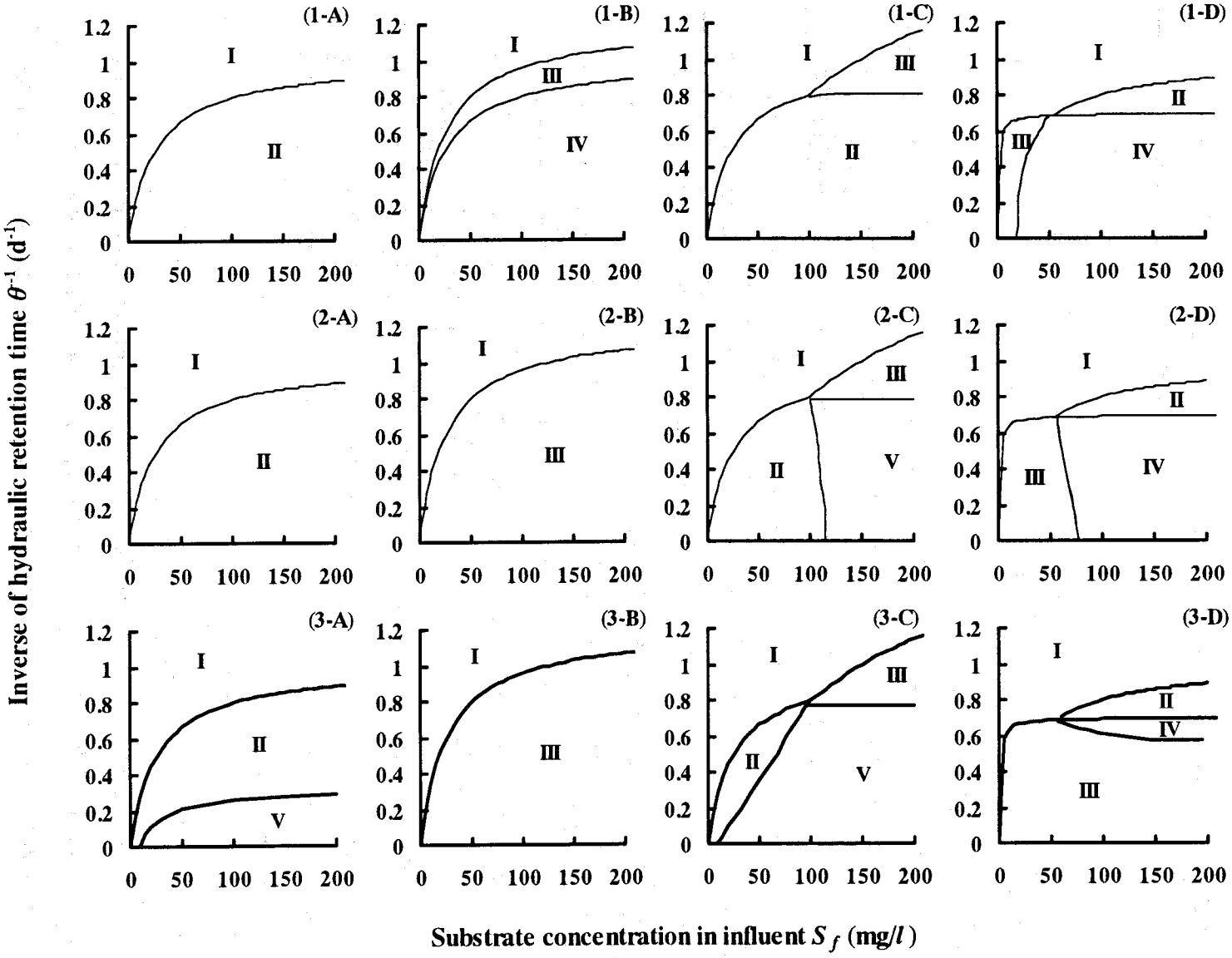

Fig. 4.5. Operational diagrams in the 12 cases. In region I, both bacterial strains are washed out. In region II, bacterial strain 1 is washed out. In region III, bacterial strain 2 is washed out. In region IV, bacterial strain 1 or 2 is washed out depending on the initial conditions. In region V, the bacterial strains coexist. (1-A) - (3-D) indicate combination of cases 1-3 in Table 4.1 and cases A- D in Table 4.2.

region V is observed in cases (2-C), (3-A), and (3-C).

4-4. DISCUSSION

The results of the simulations qualitatively correspond with the conclusion of De Freitas and Fredrickson (1978), *i. e.*, it was confirmed that production of the substance which is inhibitory against its competitor gives the producer a chance to exclude its competitor under conditions where it would otherwise be excluded by the competitor. It was also indicated that production of the substance which is not only inhibitory against its competitor but also autoinhibitory allows coexistence under wide conditions where it would otherwise exclude the competitor. Further experimental studies will be needed for verification of these simulation results.

Needless to say, the small K_S value and the large μ_m value are desirable parameters to survive for bacteria. Breeding or selection of introduced bacteria tolerant against some inhibitory substances may be a possible strategy to enhance the survival of introduced bacteria. Enhanced survival of genetically engineered microorganisms (GEMs) which are resistant to some antibiotics in soil microcosms have already been observed by simultaneous addition of the antibiotics (da Gloria et al., 1995). Another strategy is breeding or selection of bacteria which produce inhibitory substances. GEMs with such ability have already been constructed as a suicide system to control the behavior of the introduced bacteria in soil (Jemsen, et al., 1993; Ramos et al., 1994). However, this strategy will need to be more thoroughly examined and discussed in terms of the problem of biohazards. Actually, an undesirable result of bioaugmentation to a soil microcosm has been also reported that a toxic metabolite produced by a GEM depressed fungal propagules and respiration of the soil microbiota (Short et al. 1991). These suggestions may not be completely conclusive but clearly point to the importance of the antagonistic effect of indigenous bacteria on the survival of introduced bacteria in activated sludge.

4-5. SUMMARY

The survival of *P. putida* BH (pBH500) and *E. coli* K-12 (pBH500) in activated sludge microcosms was studied. Three kinds of microcosms, named as intact, protozoa-free, and sterilized microcosms, were made and used for the survival studies in order to separately evaluate the general effects of protozoan predation, interaction with indigenous bacteria, and starvation. The experimental results indicated that the interaction with indigenous bacteria,

especially in the form of antagonism, was the most influential factor causing the decline of the introduced bacterial populations, and that the protozoan predation was much less influential. Thus, in total 208 indigenous bacterial strains were isolated from the microcosm, and there were 2 strains identified as antagonistic to *P. putida* BH (pBH500) and 5 to *E. coli* C600 (pBH500). Antagonistic competition of two bacterial strains in a completely mixed reactor was simulated with a modified Monod kinetics. Simulation results suggested that production of the substance which is inhibitory against its competitor gives the producer a chance to exclude its competitor under conditions in which the producer would otherwise be excluded by the competitor. It was also found that production of a substance which is not only inhibitory against the competitor but also autoinhibitory allows both bacterial strains to coexist coexistence under wide conditions in which the competitor would be otherwise excluded.

Chapter 5. Modeling and Simulations of the Behavior of Introduced Bacteria in the Activated Sludge Process

5-1. INTRODUCTION

Behavior of the introduced bacteria in activated sludge was gradually made clear by laboratory experiments (Erb et al., 1997; Mancini et al., 1987; McClure et al., 1989, 1991; Nüblein et al., 1992; Fujita et al., 1994a; Kanagawa and Mikami, 1995; Watanabe and Hino, 1996; Watanabe et al., 1998). The experiments offered general qualitative knowledge that the introduced bacteria often show the biphasic behavior (the rapid declining phase and the slow declining phase). On the other hand, the effects of bioaugmentation on the performance of the process was demonstrated by a theoretical model (Lee, 1997), however, the model did not consider the typical behavior of the introduced bacteria. For evaluating the effects of bioaugmentation, it is primarily important to make clear the general survival mechanism of the introduced bacteria. In order to develop effective strategies for maintenance of the introduced bacteria in activated sludge, more systematic knowledge is needed. Development of an appropriate model describing the survival mechanism of the introduced bacteria would provide understanding of how operational parameters of the process affect their survival and how long they can ultimately survive in given conditions.

In Chapter 2, the fundamental concept of the general mechanism of the survival of introduced bacteria in activated sludge was proposed. In Chapter 3, a sub-model which describes the adsorption process of bacterial cells to flocs was developed. In Chapter 4, a sub-model which describes the antagonistic relationships between indigenous and introduced bacteria in activated sludge was developed. In this chapter, the sub-models were synthesized for developing a dynamic model describing the general mechanism of the introduced bacteria

in activated sludge.

5-2. MODEL DEVELOPMENT

Model concept The fundamental concept for modeling the behavior of introduced bacteria in activated sludge was reillustrated in Fig. 5.1. Following assumptions were made for mathematical modeling:

(i) A bacterium is introduced in the suspended form into a conventional activated sludge process, in which a indigenous bacterium in activated sludge establishes the steady-state. The introduced bacterium (bacterial strain 1) and the indigenous bacterium (bacterial strain 2) compete for a common substrate (a pollutant). Behavior of the introduced and the indigenous bacteria is independent of other indigenous microorganisms.

(ii) Each bacterial population is divided into two sub-populations, as mentioned in Chapter 2, which are populations adsorbed onto flocs ($\mathbf{X_f}$) and in the suspended form ($\mathbf{X_s}$). Secondary settling tanks ideally functions, and outflows of $\mathbf{X_f}$ and $\mathbf{X_s}$ from the activated sludge process occur by sludge waste and discharge of effluent, respectively.

(iii) The adsorption process of the bacterial cells onto flocs is expressed by the modified Freundlich model developed in Chapter 3. For simplification, the lag time was assumed to be zero.

(iv) The indigenous bacterium produces an inhibitory substance. Their growth is expressed by the modified Monod equation developed in Chapter 4. The production rate of the inhibitory substance is proportional to the growth rate of its producer, and neither bacterium consumes the inhibitory substance.

Mass balances of the introduced and the indigenous bacteria, the common substrate, and the inhibitory substance in the activated sludge are expressed by following equations.

[Mass balances of the introduced bacterium in activated sludge]

$$X_1 = X_{1f} + X_{1s} \qquad (5.1)$$

$$\frac{dX_{1f}}{dt} = \mu_1 X_{1f} + A_1 X_{1s}^{m} - D_1 X_{1f} - \frac{1}{\theta_c} X_{1f} \qquad (5.2)$$

$$\frac{dX_{1s}}{dt} = \mu_1 X_{1s} - A_1 X_{1s}^{m} + D_1 X_{1f} - \frac{1}{\theta} X_{1s} \qquad (5.3)$$

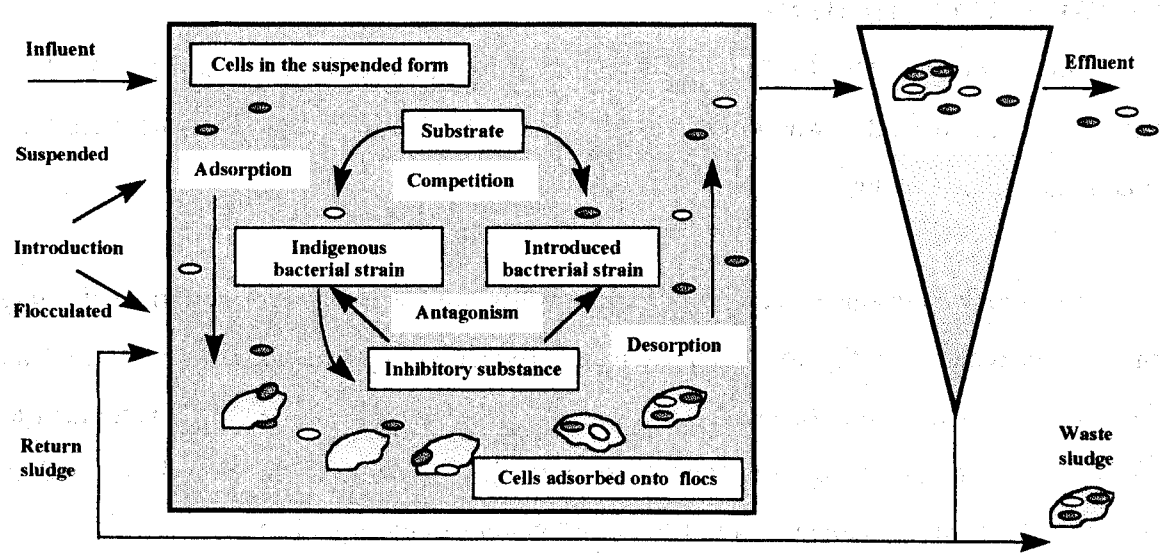

Fig. 5.1. Behavior of introduced bacteria in the activated sludge process. Following assumptions were made for modeling; (i) an introduced bacterium and an indigenous bacterium compete for a common substrate, and their behavior is independent of other indigenous microorganisms in the process; (ii) each bacterial population is divided into two sub-populations consisted of the adsorbed on flocs and in the suspended form; (iii) adsorption of the bacterial strains onto flocs is expressed by a modified Freundlich model: (iv) the indigenous bacterium produces an inhibitory substrate against the introduced bacterium and itself and their growth is expressed by a modified Monod equation.

[Mass balances of the indigenous bacterium in activated sludge]

$$X_2 = X_{2f} + X_{2s} \qquad (5.4)$$

$$\frac{dX_{2f}}{dt} = \mu_2 X_{2f} + A_2 X_{2s}^{m} - D_2 X_{2f} - \frac{1}{\theta_c} X_{2f} \qquad (5.5)$$

$$\frac{dX_{2s}}{dt} = \mu_2 X_{2s} - A_2 X_{2s}^{m} + D_2 X_{2f} - \frac{1}{\theta} X_{2s} \qquad (5.6)$$

[Mass balance of the substrate in activated sludge]

$$\frac{dS}{dt} = \frac{S_f - S}{\theta} - \frac{\mu_1 (X_{1s} + X_{1f})}{Y_1} - \frac{\mu_2 (X_{2s} + X_{2f})}{Y_2} \qquad (5.7)$$

[Mass balance of the inhibitory substance in activated sludge]

$$\frac{dI}{dt} = -\frac{I}{\theta} + p_1\mu_1(X_{1s} + X_{1f}) + p_2\mu_2(X_{2s} + X_{2f}) \qquad (5.8)$$

[Specific growth rate of the introduced bacterium]

$$\mu_1 = \frac{\mu_{m1}S}{K_{S1} + S}(1 - \frac{I}{K_{I1}}) \qquad (5.9)$$

[Specific growth rate of the indigenous bacterium]

$$\mu_2 = \frac{\mu_{m2}S}{K_{S2} + S}(1 - \frac{I}{K_{I2}}) \qquad (5.10)$$

where X, S, and I are bacterial population density, concentration of the substrate, and concentration of the inhibitory substance, respectively. S_f is substrate concentration in influent, and θ and θ_c are hydraulic retention time (HRT) and sludge retention time (SRT). A, D, and m are specific adsorption rate, specific desorption rate, and the Freundlich constant. μ_m, Y, K_S, K_I, and p are maximum specific growth rate, yield constant, half saturation constant, inhibition constant, and production constant of the inhibitory substance, respectively. Subscripts 1 and 2 are the suffix for distinguishing each bacterium and f and s refer to sub-populations adsorbed onto flocs and in the suspended form, respectively.

Model parameters Equations 5.1～5.10 contain 16 parameters in total and simulation results depend on combination of those values. Parameters concerning the bacterial adsorption ability onto flocs were estimated in Chapter 3, however, those concerning the growth/decay property of bacteria have not been determined yet. Therefore, the model parameters are assumed as listed in Table 5.1 to simulate the most possible situations of bioaugmentation studies. The most possible situation is that the introduced bacterium shows lower adsorption ability, *i.e.*, the introduced bacterium have a lower specific adsorption rate onto flocs (A). For convenience, the Freundlich constant (m) and specific desorption rate (D) of both bacteria were set 1.0 and 0.01, respectively. It was also assumed that the indigenous bacterium produces an inhibitory substance against the introduced bacterium and itself, is less sensitive to the substance (K_I), and the introduced bacterium does not produce the substance (p). In addition, it was also assumed that the introduced bacterium has a higher maximum growth rate (μ_m), a smaller half saturation constant (K_S), and a lower yield constant (Y), because

Table 5.1. Parameters of the introduced bacterium (strain 1) and indigenous bacterium (strain 2)

Introduced bacterium		Indigenous bacterium	
A_1 (d^{-1})	0.01	A_2 (d^{-1})	1.0
D_1 (d^{-1})	0.01	D_2 (d^{-1})	0.01
K_{S1} (mg/l)	75	K_{S2} (mg/l)	50
K_{I1} (mg/l)	1.7	K_{I2} (mg/l)	2.0
m_1 (−)	1.0	m_2 (−)	1.0
p_1 (−)	0	p_2 (−)	0.02
Y_1 (−)	0.5	Y_2 (−)	0.7
μ_{m1} (d^{-1})	1.8	μ_{m2} (d^{-1})	1.0

bacterial strains selected for bioaugmentation are usually isolated from microbial consortia assimilated to targeted pollutants of high concentrations as mentioned in general introduction.

5-3. SIMULATIONS

Basic survival pattern of introduced bacteria in activated sludge Simulations were carried out using the 4th order Runge-Kutta method provided by the software ISIM for Windows (Snape et al., 1995). Figure 5.2 shows a typical simulation result of behavior of the introduced bacterium in activated sludge (θ = 1.25 d, θ_c = 20 d, S_f = 200 mg/l). The bacterium (strain 1) was introduced on day 0. For convenience, 2000 mg/l of the bacteria was assumed to be corresponded to 10^9 CFU/ml.

The proposed model satisfactory described the biphasic survival pattern which consists of the rapid declining phase and the slow declining phase. The behavior of the introduced bacterium during the rapid declining phase was represented mainly by the survival of the sub-population in the suspended form, while that in the slow declining phase was represented mainly by the behavior of the sub-population adsorbed onto flocs.

The indigenous bacterial population was little affected by the introduced bacterium. The values of X_{2f}, X_{2s}, S, and I have remained stable at 6.7×10^8 CFU/ml, 3.8×10^6 CFU/ml, 69 mg/l, and 1.8 mg/l, respectively although those were disrupted for a few days after the bioaugmentation.

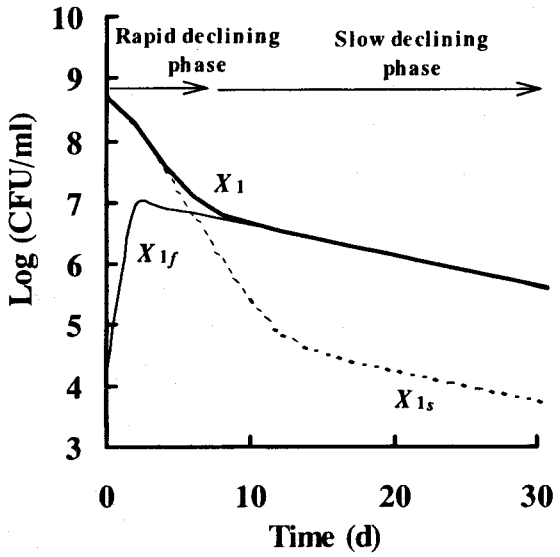

Fig. 5.2. A typical simulation result of survival of the introduced bacterium in the activated sludge process (θ=1.25d, θ_c=20d, S_f=200 mg/l).

Effect of operational parameters on behavior of introduced bacteria The influences of the inoculum size, HRT, and SRT on behavior of the introduced bacterium are shown in Fig. 5.3. As shown in Chapter 2, the larger inoculum sizes, the introduced bacterium survived at higher levels during a certain period as shown in Fig. 5.3 (A). Under longer HRT operations, the introduced bacterium survived at higher levels during the rapid declining phase as shown in Fig. 5.3 (B). The enhanced survival of the introduced bacterium with increase in HRT was explained by the enhanced survival of the sub-population in the suspended form. An increase in HRT should make the cells in the suspended form physically retainable for a longer time in the process. On the other hand, under shorter SRT operations, the introduced bacterium survived at higher levels during the slow declining phase as shown in Fig. 5.3 (C). The enhanced survival of the introduced bacterium with decrease in SRT was explained by the enhanced survival of the sub-population adsorbed onto flocs of the introduced bacterium which have the higher maximum growth rate. These simulation results indicate that the developed dynamic model with the assumed parameters could describe properly the behavior of introduced bacteria in activated sludge.

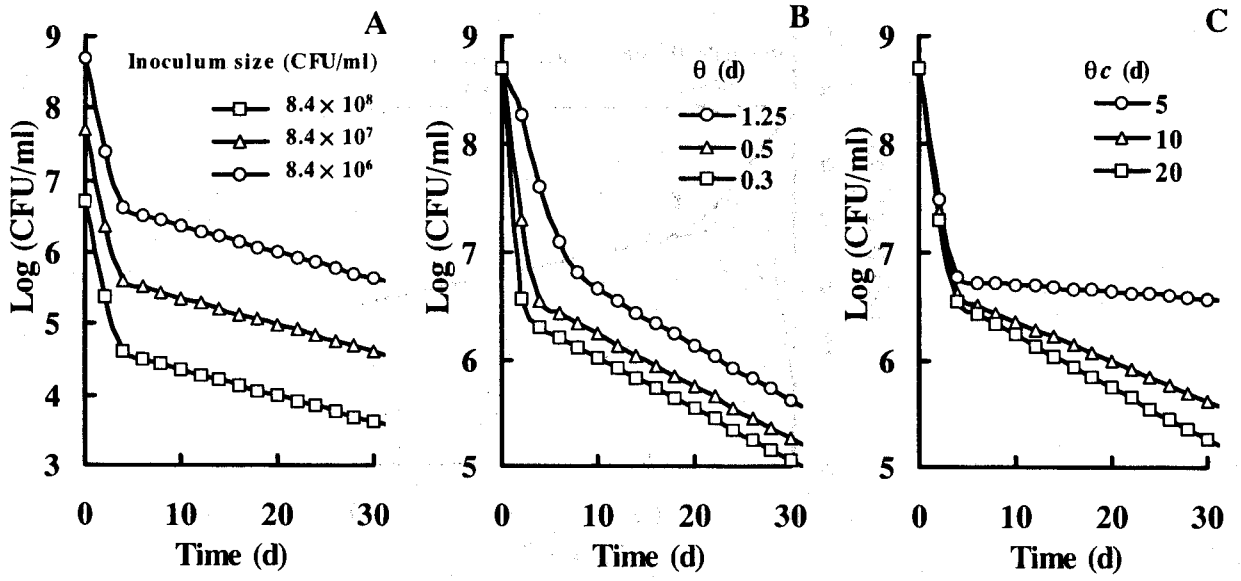

Fig. 5.3. Simulation results of behavior of the introduced bacterium in the activated sludge process under various operational conditions. The behavior of different inoculum sizes (θ = 0.5 d, θ_c = 10 d) (A), at three HRTs (inoculum size = 8.4×10^8 CFU/ml, θ_c = 10 d) (B), and three SRTs (inoculum size = 8.4×10^8 CFU/ml, θ = 0.5 d) (C). All simulations were carried out at S_f = 200 mg/*l*.

Successful bioaugmentation to activated sludge SRT and substrate concentration in influent are important factors to control the ultimate survival level of the introduced bacteria. At a short SRT operation (θ_c = 4 d, θ = 0.5 d, S_f = 200 mg/*l*), the introduced bacterium showed better survival than the indigenous bacterium, and its behavior was divided into 3 phases (Fig. 5.4). First, the population of the introduced bacterium rapidly decreased (rapid declining phase) and the substrate removal was temporarily enhanced. Subsequently, the introduced bacterium gradually increased in its population (succession phase) and it remained stable at 3.6×10^8 CFU/ml after *c.a.* 150 d (stable phase). The indigenous bacterium showed drastic decrease on its population in the stable phase. Before bioaugmentation, only 45% of the substrate was removed, however, more than 90% of the removal was performed in the stable phase, indicating the bioaugmentation succeeded.

The effect of SRT of the activated sludge process on the bacterial populations in the stable phase is shown in Fig. 5.5 (θ = 0.5 d, S_f = 200 mg/*l*). The effect of bioaugmentation was observed at less than *c.a.* 4 d of SRT. With the parameters listed in Table 5.1, short SRT operations are advantageous to the introduced bacterium because the indigenous bacterium has a lower maximum growth rate, consequently, decrease in the indigenous bacterial

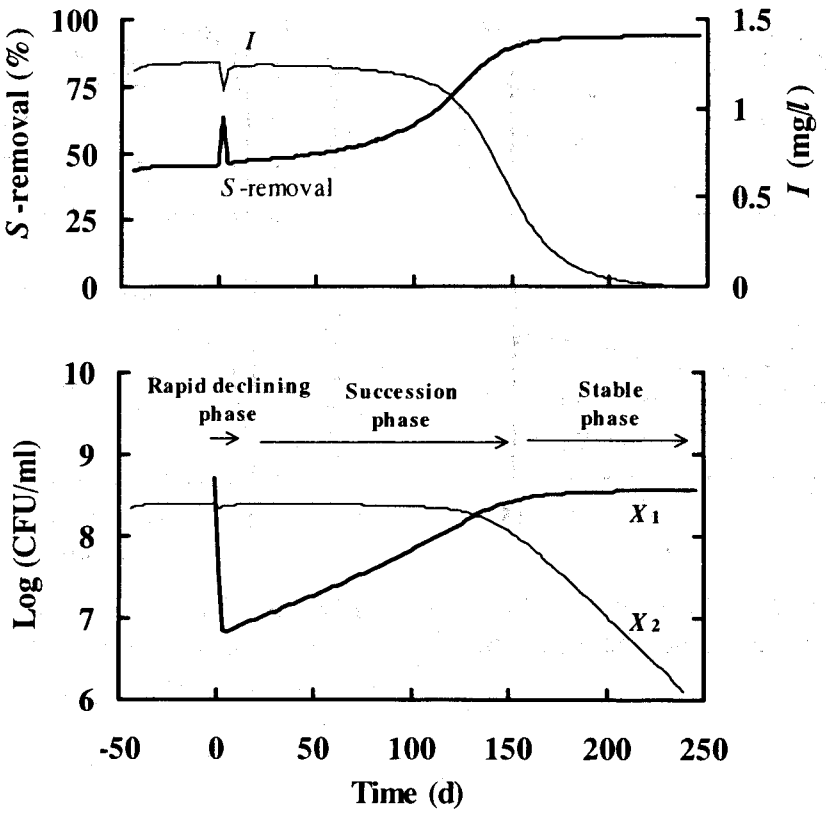

Fig.5.4. A successful operation of the bioaugmented activated sludge process (inoculum size = 8.4×10^8 CFU/ml, $\theta = 0.5$ d, $\theta_c = 4$ d, $S_f = 200$ mg/*l*).

population caused decrease in the concentration of the inhibitory substance. Under 3 d and 5 d of SRT, the stable phase was established at about 20 d and 50 d, respectively.

Figure 5.6 shows the effect of the substrate concentration in influent on the bacterial populations (θ_c = 10 d, θ = 0.5 d). The effect of bioaugmentation was observed at lower substrate concentrations than *c.a.* 120 mg/*l* because the concentration of the inhibitory substrate decreases with the substrate concentration in influent.

These simulation results suggested that bioaugmentation is possible to be successful if the activated sludge process are operated under proper conditions. However, it was also suggested that it needs a long time more than a few months to establish the stable phases. HRT has a little effect on the substrate removal and bacterial populations in the stable phase although it affects the periods of the rapid declining and the succession phases (data not shown).

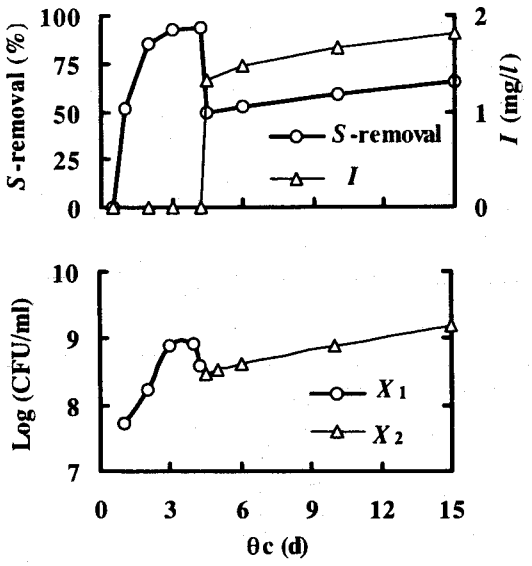

Fig. 5.5. Effect of SRT on the bacterial populations in the stable phase (θ = 0.5 d, S_f = 200 mg/*l*).

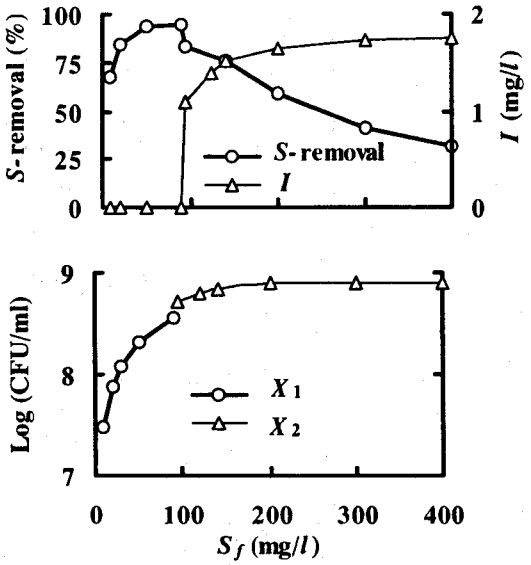

Fig. 5.6. Effect of substrate concentration in influent on the microbial populations in the stable phase (θ = 0.5 d, θ_c = 10 d).

5-4. DISCUSSION

In many models used to describe activated sludge processes, the microbial populations is considered as a whole, and no distinctions are made between the different microorganisms that exist in activated sludge. Curds (1973) developed a model that considers the microbial ecology in activated sludge, in which the interactions between the bacteria and the protozoa are discussed using Lotka-Volterra model (Lotka, 1920). Lee (1997) developed a bioaugmented activated sludge model that considers the behavior of an introduced bacterium and an indigenous bacterium which is representative of whole indigenous microorganisms, however, survival of the introduced bacterium and the microbial interactions were not accounted. In comparison with those models, the developed dynamic model in this chapter contains two important factors affecting the behavior of introduced bacteria in activated sludge, *i.e.*, the adsorption process of bacterial cells onto flocs and the interactions between introduced and indigenous bacteria.

The developed model could successfully simulate similar trends to the experimental data shown in Chapters 1 and 2. Thus, it was suggested that successful bioaugmentation needs proper operational conditions of the process. However, those results depend on the model parameters and different simulation results should be obtained from different parameters.

Therefore, the mathematical modeling cannot replace experimental techniques and data, however, it can prove to be an excellent way of presenting complex phenomena in an efficient form and the model will help in understanding the real behavior of introduced bacteria in activated sludge.

It was also suggested that the behavior of the introduced bacterium mainly depends on the initial adsorption of the introduced cells onto flocs and its growth on flocs. Floc-forming bacteria, like *Sphingomonas paucimobilis* 551 (Fujita et al., 1991), with higher adsorption ability onto flocs (larger A value, smaller D value, and/or larger m value) than indigenous bacteria have a possibility to win the competition depending on the operational conditions even if they have lower growth ability (smaller μ_m value, larger K_S value, and/or smaller K_I value). Thus, floc formation or adsorption onto flocs may bring many advantages to bacteria such as protection against protozoan predation, resistance to toxins, provision for reserve energy sources (Bar-Or 1990; Bossier and Verstraete, 1996) although the growth rate of the sub-population adsorbed on flocs was assumed to be equivalent to that in the suspended form. Contrary, bacteria with much higher growth ability than indigenous bacteria have also a possibility to win the competition even if they have lower adsorption ability onto flocs. For example, similar behavior of an introduced bacterium to the triphasic behavior consisting of the rapid declining, the succession, and the stable phases shown in Fig. 5.4 was reported by Kanagawa and Mikami (1995) although the stable phase was observed after only 1 week. They introduced a non-floc-forming bacterium, which degrades *O*, *O*-dimetyl phosphorodithioate (DMDTP), into a laboratory activated sludge process, and the activated sludge developed the stable population of the introduced bacterium and DMDTP-degradation activity. As reasons for the successful bioaugmentation, it was suggested that there would be no competitor degrading DMDTP in the activated sludge and the introduced cells have survived by adsorbing themselves onto flocs during the rapid declining phase and the succession phase, and have grown to exhibit the degradation activity.

For further studies, some detailed models will be helpful, which consider the bacterial metabolism and the interactions with other microorganisms as shown in Fig. 5.7. It is well known that bacterial populations expressed by the Lotka-Volterra model which describes a predator-prey relationship exhibit stable steady states, unstable steady states, and oscillatory behavior (Lotka, 1920). Growth of bacteria is often inhibited at high concentrations of their own cells, metabolites, and substrates (Han and Levenspiel, 1988; Simkins and Alexander, 1984). Thus, bacterial degradation of some kinds of substrates is drastically influenced by the

presence of other substrates (Chang and Cohen, 1995; Fujita et al., 1995; Wiggins and Alexander, 1988).

Considerations of spatially heterogeneity of bacterial populations and substrate concentrations in flocs would be also helpful for further applications (Fig. 5.8). It is known that bacteria in flocs and biofilms compete for not only substrates but also the limited space (Benefield and Molz, 1983; Furumai and Rittmann, 1994).Wastewater treatment processes successfully utilizing flocculated or aggregated biomass such as activated sludge processes, trickling filters, and rotating biological contactors, are regarded more robust than well mixed processes (Rittmann, 1982; Tyagi et al., 1996). This is often explained by protection of bacteria in inner layers of the floc and/or the biofilm from the harsh outer environment. There are, however, few quantitative studies of the population dynamics in flocs and/or biofilms using adequate mathematical models and simulations (Wanner and Gujer, 1986; Rittmann, 1992; Furumai and Rittmann, 1994). Those models were mainly used for evaluation of the spatial distribution of bacteria with different nutritional requirements, such as heterotrophic and autotrophic bacteria.

Furthermore, many academic and industrial institutes are now working on breeding genetically engineered microorganisms (GEMs) and the application of them to wastewater treatment processes. The risk of accidental release to natural environments or the planned introduction of GEMs emphasizes the urgent need for a more detailed knowledge of their behavior. Application of recombinant genes concerning production of fimbriae or bioflocculants will change the adsorption/desorption parameters, A, D, and m, of the bacterial strains. While, application of genes concerning the degradation of targeted pollutants or the tolerance to inhibitory substances will change the parameters concerning the growth/decay property, μ_m, K_s, and K_i, of the bacterial strains (Fig. 5.9). In case that plasmids are used as a vector of such recombinant genes, the plasmid stability in the host strain should be considered. In general, plasmid stability means the ability of GEMs to maintain their recombinant plasmids unchanged during growth, *i.e.*, stable expression of their phenotypic expression. It has been generally observed that plasmid-free cells appear after a certain lag time in the continuous culture of plasmid-harboring cells under non selective conditions (Fujita et al., 1991; Pickup et al., 1991; Shohan and Demain, 1991; Simonsen, 1991). Subsequently, the plasmid-free cells increased as the cultivation proceeded. It was also pointed that transfer of genes between introduced and indigenous bacteria will play an important role in the bioaugmentation strategy. Possibility of the transfer of recombinant plasmids in activated

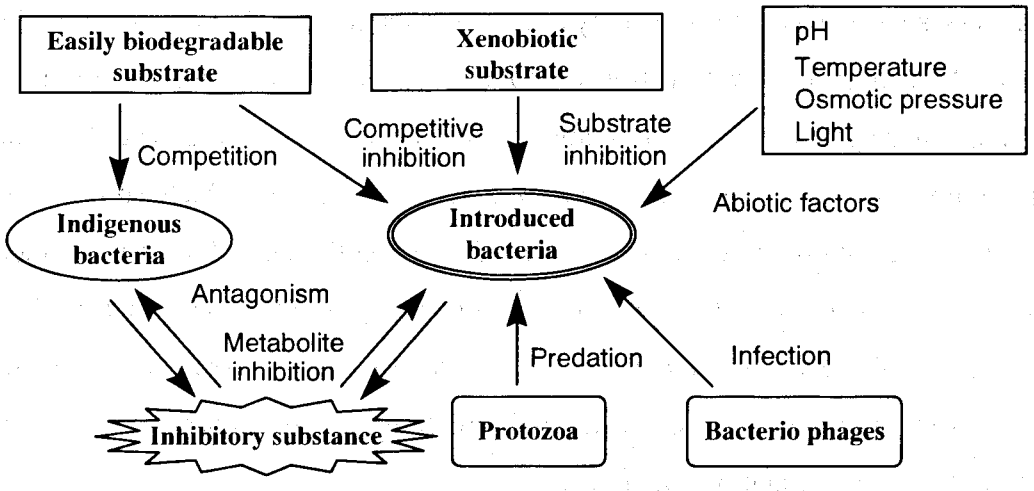

Fig. 5.7. Factors affecting the behavior of introduced bacteria in activated sludge.

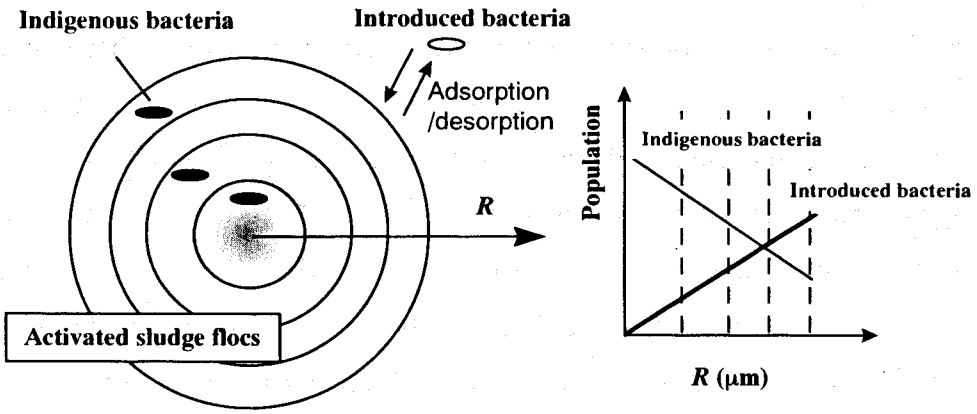

Fig.5.8. The fine differencing of the spherical activated sludge floc.

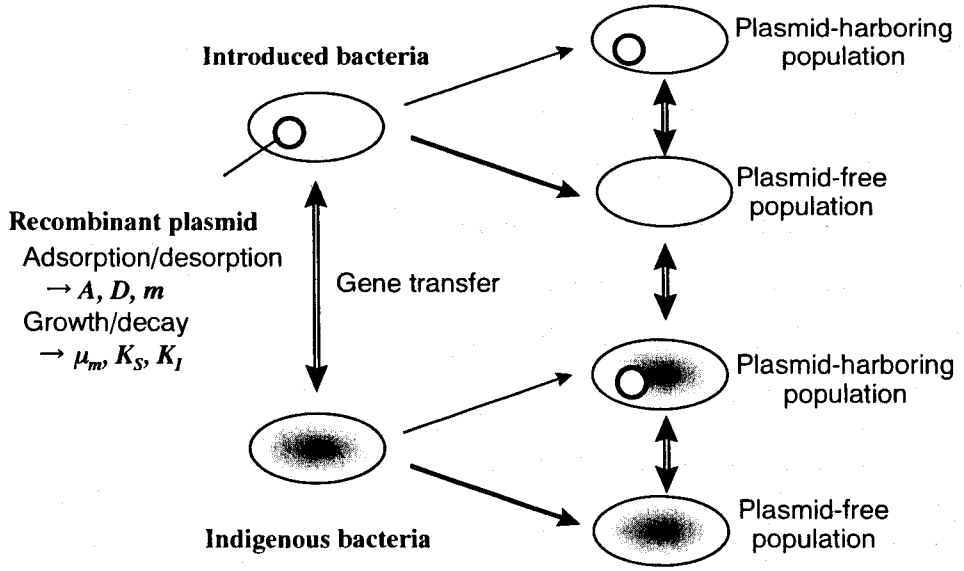

Fig. 5.9. Dissemination of recombinant DNAs by gene transfer.

sludge processes has been demonstrated by a few research groups (Focht et al., 1996; Fujita et al., 1993b; Mancini et al., 1987; Top et al, 1990). It could be interesting to insert specific genes coding for degradation and flocculation on a transferable plasmid. These recombinant plasmids could spread into the indigenous bacterial community, which could lead to an overall better flocculation and degradation of recalcitrant pollutants. However, the increasing likelihood of introduction of GEMs into activated sludge processes has emphasized the lack of knowledge on the gene transfer, and concerns have been expressed recombinant genes could be transferred to the indigenous community with undesirable consequences, such as the disruption of bacterial population dynamics.

5-5. SUMMARY

A dynamic model describing the general behavior of introduced bacteria in activated sludge were developed with following assumptions; (i) an introduced bacterium and an indigenous bacterium compete for a common substrate, and their behavior is independent of other indigenous microorganisms in the process; (ii) each bacterial population is divided into two sub-populations consisted of the adsorbed on flocs and in the suspended form; (iii) adsorption of the bacterial strains onto flocs is expressed by a modified Freundlich model: (iv) the indigenous bacterium produces an inhibitory substrate against the introduced bacterium and itself and their growth is expressed by a modified Monod equation. The simulation results indicated that the proposed dynamic model can describe the typical behavior of introduced bacteria in activated sludge, and further model development was discussed.

5-6. NOMENCLATURE

A	Specific adsorption rate (d^{-1})
D	Specific desorption rate (d^{-1})
I	Inhibitory substance concentration (mg/l)
K_S	Half saturation constant (mg/l)
K_I	Inhibition constant (mg/l)
m	Freundlich constant (−)
p	Production constant of the inhibitory substance (−)
S	Substrate concentration (mg/l)
S_f	Substrate concentration in influent (mg/l)
t	Time (d)

X	Population density of the bacterium in activated sludge (mg/l; 2000 mg/l of X corresponds to 10^9 CFU/ml)
Y	Yield constant (−)
μ_m	Maximum specific growth rate (d^{-1})
θ	SRT: sludge retention time (d)
θ_c	HRT: Hydraulic retention time (d)

Subscripts

f	Sub-population attached onto flocs
s	Sub-population in the suspended form
1	Introduced bacterium
2	Indigenous bacterium

Summary and Conclusion

For establishment of effective bioaugmentation strategies, this study focused on behavior of introduced bacteria in the activated sludge process.

First, a genetically engineered bacterial strain, which was designed to exhibit high phenol-degrading activity, was introduced into a model activated sludge process treating phenolic wastewater. The experimental results suggested that bioaugmentation can be a useful means of improving treatment of the activated sludge process, however, its effect was gradually lost as the introduced bacterial population declined. The declining of the introduced bacterial strain in the activated sludge process was a biphasic pattern, *i.e.*, the rapid declining phase and the slow declining phase (Chapter 1).

It was suggested that operational conditions of the activated sludge process such as hydraulic retention time (HRT) and sludge retention time (SRT) affect the behavior of introduced bacteria in activated sludge. A concept for modeling the behavior of the introduced bacteria was proposed with a following fundamental assumption; (I) the total population of the introduced bacteria in activated sludge should be given by the sum of two sub-populations: that of ones adsorbed on sludge flocs ($\mathbf{X_f}$) and that of ones suspended in liquid ($\mathbf{X_s}$). Factors affecting the behavior of the introduced bacteria were discussed and it was concluded that the adsorption/desorption onto/from flocs and growth/decay of the introduced bacteria are the most important factors (Chapter 2).

The adsorption/desorption process of introduced bacterial cells onto activated sludge flocs was investigated in Chapter 3. The equilibrium of the bacterial cells obeyed the Freundlich isotherm and an adsorption kinetics could simulate the adsorption process.

While, it was also suggested that the interaction with indigenous bacteria with antagonistic activity, which produce lethal substances, is the most influential factor affecting growth/decay of the introduced bacteria in activated sludge. Simulation studies of the

antagonistic competition of two bacterial strains, using a modified Monod kinetics with a numerical technique, suggested that inhibitory substances have important roles in maintenance of bacterial species-diversity in ecosystems (Chapter 4).

Finally, based on the model concept proposed in Chapter 2 and the sub-models developed in Chapters 3 and 4, a dynamic model describing the general mechanism of the behavior of introduced bacteria in the activated sludge process was developed in Chapter 5. Following major assumptions were added for simulations; **(II)** an introduced bacterium and an indigenous bacterium compete for a common substrate, and those behavior is independent of other microorganisms in activated sludge; **(III)** the adsorption process of the bacterial cells onto flocs is described by the modified Freundlich model; **(IV)** the indigenous bacterium produces an inhibitory substance against the introduced bacterial strain and itself, and their growth is expressed by the modified Monod model.

The proposed model satisfactory described the biphasic survival pattern which consists of the rapid declining phase and the slow declining phase. The behavior of the introduced bacterium during the rapid declining phase was represented mainly by the survival of the $\mathbf{X_s}$, sub-population in the suspended form, while that in the slow declining phase was represented mainly by the behavior of $\mathbf{X_f}$, the sub-population adsorbed onto flocs. The introduced bacterium should be in the suspended form soon after introduction, therefore it can be easily withdrawn into the effluent, resulting in the fast drop of $\mathbf{X_s}$ in the rapid declining phase, therefore, long HRT operations enhances the survival of $\mathbf{X_s}$. On the other hand, its survival in the slow declining phase depends on the withdrawn of waste sludge and growth/decay rates of $\mathbf{X_f}$. In case that the introduced bacterial strain has a higher specific growth rate than indigenous bacterial strains, short SRT operations enhances the survival of $\mathbf{X_f}$ in the slow declining phase.

Recently, there have been much concern regarding the intentional release (bioaugmentation) of desirable bacteria including genetically engineered bacteria to natural ecosystems for bioremediation (Pikup et al., 1991; Harvey, 1993; Krumme et al., 1994; Lindow et al., 1989; Tiedje et al., 1989). The biphasic behavior, which consists of rapid declining and slow declining phases, of introduced bacteria was observed not only in activated sludge but also in anaerobic digestion (Kearney et al., 1993), aerated lagoon (Mezrioui and Baleux, 1994), soil (Macnaughton et al., 1992; Recorbet et al., 1993), and aquatic microcosms (Humpheson et al., 1998; Inamori et al., 1992; Kotewara and Alexander, 1990; Tanaka et al., 1995). Some researchers also suggested that the introduced bacterial strain in natural

environments consists of two sub-populations which show different behavior each other although the sub-populations were not clearly defined (Inamori et al., 1992; Matthess and Pekdeger, 1986; Peterson and Ward, 1986; Recorbet et al., 1993; Tanaka et al., 1995). It may be because the bacterial population adsorbed on small particles in such environments shows different behavior from that in the suspended or free-moving form. For example, 15-30 % of the total bacterial count has been commonly found adsorbed onto particles, although values as high as 70-95 % have been observed (Pickup et al., 1991). Therefore, with some modifications, the model developed in this study will be applicable as a reasonable tool to predict the fate of introduced bacteria in natural environments as well as the activated sludge process.

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References

Amy, P. S., Hermid, D., and Hiatt, H. D. (1989) Survival and detection of bacteria in aquatic environment. *Appl. Environ. Microbiol.*, **55,** 788-793.

Appleyard, P. K. (1954) Segregation of new lysogenic types during growth of a doubly lysogenic strain derived from *Escherichia coli* K12. *Genetics*, **39,** 440-452.

Aris, R. and Humphrey, A. E. (1977) Dynamics of a chemostat in which two organisms compete for a common substrate. *Biotechnol. Bioeng.*, **19,** 1375-1386.

Barcina, I., Arana, I., Astorga, A. F., Iriberri, J., and Egea, L. (1996) Survival strategies of plasmid-carrier and plasmidless *Escherichia coli* strains under illuminated and non-illuminated conditions, in a fresh water ecosystem. *J. Appl. Bacteriol.*, **73,** 229-236.

Barcina, I., Lebaron, P., and Vives-Rego, J. (1997) Survival of allochthonous bacteria in aquatic system: A biological approach. *FEMS Microbiol. Ecol.*, **23,** 1-9.

Bar-Or, Y. (1990) The effect of adhesion on survival and growth of microorganisms. *Experientia*, **46,** 823-826.

Benefield, L. and Molz, F. (1983) A kinetic model for the activated sludge process which considers diffusion and reaction in the microbial floc. *Biotechnol. Bioeng.*, **25,** 2591-2615.

Bieszkiewicz, E. and Fiutowska, L. (1991) The effect of aromatic compounds on the work of activated sludge. *Acta Microbiologica Polonica*, **40,** 177-185.

Bolton, H., Fredrickson, J. K., Bentjen, S. A., Workman, D. J., Li, S. W., and Thomas, J. M. (1991) Field calibration of soil-core microcosms: Fate of a genetically altered rhizobacterium. *Microb. Ecol.*, **21,**163-173.

Bossier, P. and Verstraete, W. (1996) Triggers for microbial aggregation in activated sludge? *Appl. Microbiol. Biotechnol.*, **45,** 1-6.

Brettar, I., Gonzalez, M. I. R., Ramos, J. L., and Hofle, M. G. (1994) Fate of *Pseudomonas*

putida after release into lake water mesocosm : Different survival mechanisms in response to environmental conditions. *Microb. Ecol.*, **27,** 99-122.

Bright, J. B. and Fletcher, M. (1983) Amino acid assimilation and respiration by attached and free-living populations of marine *Pseudomonas* sp. *Microb. Ecol.*, **9,** 215-226.

Brown, V. R. and Knapp, J. S. (1990) The effect of withdrawal of morpholine from the influent and its reinstatement on the performance and microbial ecology of a model activated sludge plant treating a morpholine-containing influent. *J. Appl. Bacteriol.*, **69,** 43-53.

Cardinal, L. J. and Stenstrom, M. K. (1991) Enhanced biodegradation of polyaromatic hydrocarbons in the activated sludge process. *Res. J. WPCF*, **63,** 950-957.

Chang, H. and Cohen, L. A. (1995) Model for the cometabolic biodegradation of chlorinated organics. *Environ. Sci. Technol.*, **29,** 2357-2367.

Curds, C. R. (1973) A theoretical study of factors influencing the microbial population dynamics of the activated-sludge process-I. *Wat. Res.*, **7,** 1269-1284.

da Gloria, R., de Oliveira, B., Wolters, A. C., and van Elas, J. D. (1995) Effects of antibiotics in soil on the population dynamics of transposon Tn5 carrying *Pseudomonas fluorescense*. *Plant and Soil*, **175,** 323-333.

De Freitas, M. J. and Fredrickson, A. G. (1978) Inhibition as a factor in the maintenance of the diversity of microbial ecosystems. *J. Gen. Microbiol.*, **106,** 307-320.

Diab, S. and Shilio, M. (1988) Effect of adhesion to particles on the survival and activity of *Nitrosomonas* sp. and *Nitrobacter* sp. *Arc Microbiol.*, **150,** 387-393.

Dopazo, C. P., Lemos, M. L., Loderrios, C., Bolinches, J., Barja, J. L., and Toranzo, A. E. (1988) Inhibitory activity of antibiotic-producing marine bacteria against fish pathogens. *J. Appl. Bacteriol.*, **65,** 97-101.

Espinosa, L. M. and Stephenson, T. (1996) Grease bioaugmentation: is bioaugmentation more effective than natural populations for strat-up? *Wat. Sci. Tech.*, **34,** 303-308.

Erb, R. W., Eichner, A. E., Wagner-Dobler, I., and Timmis, K. N. (1997) Bioprotection of microbial communities from toxic phenol mixtures by a genetically designed Pseudomonad. *Nat. Biotech.*, **15,** 378-382.

Farrah, S. R. and Bitton, G. (1983) Bacterial survival and association with sludge flocs during aerobic and anaerobic digestion of wastewater sludge under laboratory conditions. *Appl. Environ. Microbiol.*, **45,** 174-181.

Figurski, D. and Helinski, D. R. (1979) Replication of an origin-containing derivative of

plasmid RK2 dependent on a plasmid function provided in trans. *Proc. Natl. Acad. Sci. USA*, **76,** 1648-1652.

Focht, D. D., Searles, D. B., and Koh, S. C. (1996) Genetic exchange in soil between introduced chrolobenzoate degraders and indigenous biphenyl degraders. *Appl. Environ. Microbiol.*, **62,** 3910-3913.

Fujii, T., Takeo, M., and Maeda, Y. (1997) Plasmid-encoded genes specifying aniline oxidation from *Acinetobacter* sp. strain YAA. *Microbiology*, **143,** 93-99.

Fujita, M., Ike, M., and Hashimoto, S. (1991) Feasibility of wastewater treatment using genetically engineered microorganisms. *Wat. Res.*, **25,** 979-984.

Fujita, M., Ike, M., and Kamiya, T. (1993a) Accelerated phenol removal by amplifying the gene expression with a recombinant plasmid encoding catechol 2, 3-oxygenase. *Wat. Res.*, **27,** 9-13.

Fujita, M., Ike, M., and Suzuki, H. (1993b) Screening of plasmid from wastewater bacteria. *Wat. Res.*, **27,** 949-953.

Fujita, M. and Ike, M. (1994) Wastewater treatment using genetically engineered microorganisms., pp.141-164, Technomic Publishing, Lancaster, USA.

Fujita, M., Ike, M., and Uesugi, K. (1994a) Operation parameters affecting the survival of genetically engineered microorganisms in activated sludge processes. *Wat. Res.*, **28,** 1667-1672.

Fujita, M., Iwahori, K., and Taki, H. (1994b) A novel methods of enumerating *Nocardia amarae* in foaming activated sludge. *J. Ferment. Bioeng.*, **77,** 674-678.

Fujita, M., Ike, M., Hioki, J., Kataoka, K., and Takeo, M. (1995) Trichloroetylene degradation by genetically engineered bacteria carrying cloned phenol catabolic genes. *J. Ferment. Bioeng.*, **79,** 100-106.

Fujita, M., Iwahori, K., Kawaguchi, Y., and Sakai, Y. (1996) A survey of operating problems associated with sewage treatment plants in Japan. *Japanese J. Wat. Biol.*, 32, 257-269.

Furumai, H. and Rittmann, B. E. (1994) Evaluation of multiple-species biofilm and floc processes using a simplified aggregate model. *Wat. Sci. Tech.*, **29,** 439-446.

Gauthier, M. J., and Flatas, G. F. (1976) Antibacterial activity of marine violet-pigmented *Alteromonas* with special reference to the production of brominated compounds. *Can. J. Microbiol.*, **22,** 1612-1619.

Gealt, M. A., Chai, M. D., Alppert, K. B., and Boyer, J. C. (1985) Transfer of plasmids

pBR322 and pBR325 in wastewater from laboratory strains of *Escherichia coli* to bacteria indigenous to the waste disposal system. *Appl. Environ. Microbiol.*, **49,** 836-841.

Gonzalez, J. M., Sheerr, E. B., and Sherr, B. F. (1990) Size-selective grazing on bacteria by natural assemblages esturine flagellates and ciliates. *Appl. Environ. Microbiol.*, **56,** 583-589.

Gujer, W., and Kappeler, J. (1992) Modeling population dynamics in activated sludge system. *Wat. Sci. Tech.*, **25,** 93-103.

Gujer, W., Henze, M., Mino, T., Matsuo, T., Wentzel, M. C., and Maris, G. V. R. (1995) The activated sludge model No. 2: biological phosphorus removal. *Wat. Sci. Tech.*, **31,** 1-11.

Gurijala, K. R., and Alexander, M. (1990) Explanation for the decline of bacteria introduced into lake water. *Microb. Ecol.*, **20,** 231-244.

Han, K. and Levenspiel, O. (1988) Extended Monod kinetics for substrate, product, and cell inhibition. *Biotechnol. Bioeng.*, **32,** 430-437.

Harvey, R. W. (1993) Fate and transport of bacteria injected into aquifers. *Curr. Opin. Biotechnol.*, **4,** 312-317.

Hashimoto, S. and Fujita, M. (1987) Identification of three phenol-degrading microorganisms isolated from activated sludge and their characteristics. *J. Japan Sew. Wks.*, **9,** 655-660. (in Japanese)

Heitkamp, M. A., Kane, J. F., Morris, P. J. L., Bianchini, M, Hale, M. D., and Bogosian, G. (1993) Fate in sewage of a recombinant *Escherichia coli* K-12 strain used in the commercial production of bovine somatoropin. *J. Ind. Microbiol.*, **11,** 243-252.

Henze, M., Grady, C. P. L., Gujer, W., Marais, G. V. R., and Matsuo, T. (1987) A general model for single sludge wastewater treatment systems. *Wat. Res.*, **21,** 505-515.

Hess, T. F., Schmidt, S. K., Silverstein, J., and Howe, B. (1990) Supplemental substrate enhancement of 2,4-dinitrophenol mineralization by a bacterial consortium. *Appl. Environ. Microbiol.*, **56,** 1551-1558.

Hess, T. F., Silverstein, J., and Schmidt, S. K. (1993) Effect of glucose on 2,4-dinitrophenol degradation kinetics in sequencing batch reactors. *Wat. Environ. Res.*, **65,** 73-81.

Humpheson, L., Adams, M. R., Anderson, W. A., and Cole, M. B. (1998) Biphasic thermal inactivation kinetics in *Salmonella enteritidis* PT4. *Appl. Environ. Microbiol.*, **64,** 459-464.

Inamori, Y., Murakami, K., Sudo, R., Kurihara, Y., and Tanaka, N. (1992) Environmental assessment method for field release of genetically engineered microorganisms using microcosm system. *Wat. Sci. Tech.*, **26,** 2161-2164.

Iwasaki, K., Uchiyama,. H., and Yagi, O. (1993) Survival and impact of genetically engineered *Pseudomonas putida* harboring mercury resistance gene in aquatic microcosms. *Biosci. Biotechnol. Biochem.*, **57,** 1264-1269.

Jacob, A. E. and Grinter, N. J. (1975) Plasmid RP4 as a vector replicon in genetic engineering. *Nature,* **255,** 504-506.

Jemsen, L. B., Ramos, J. L., Kaneva, Z., and Molin, S. (1993) A substrate-dependent biological containment system for *Pseudomonas putida* based on the *Escherichia coli gef* gene. *Appl. Environ. Microbiol.*, **59,** 3713-3717.

Kanagawa, T. and Mikami, E. (1995) Survival of a non flocculating bacterium, *Thiobacillus thioparus* TK-1, inoculated to activated sludge. *Wat. Res.*, **29,** 2751-2754.

Kanuf, V. C. and Nester, E. W. (1982) Wide host range cloning vectors: a cosmid clone bank of an *Agrobacterium* Ti plasmid. *Plasmid*, **8,** 45-54.

Kearney, T. E., Larkin, M. J., and Levett, P. N. (1993) The effect of slurry storage and anaerobic digestion on survival of pathogenic bacteria. *J. Appl. Bacteriol.*, **74,** 86-93.

Klein, T. M. and Alexander, M. (1986) Bacterial inhibitors in lake water. *Appl. Environ. Microbiol.*, **52,** 114-118.

Kotewara, R. G. and Alexander M. (1990) Explanation for the decline of bacteria introduced into lake water. *Microbial. Ecol.*, **20,** 231-244.

Krumme, M. L., Smith, R. L., Egestorff, J., Thiem, S. M., Tiedje, J. M., Timmis, K. N., and Dwyer, D. F. (1994) Behavior of pollutant degrading microorganisms in aquifers: predictions for genetically engineered organisms. *Environ. Sci. Technol.*, **28,** 1134-1138.

Kurane, R., Toeda, K., Takeda, K., and Suzuki, T. (1986) Culture conditions for production of microbial flocculant by *Rhodococcus erythropolis. Agric. Biol. Chem.*, **50,** 2309-2313.

Lee, C. (1997) Model of bacterial-supplement kinetics. *J. Environ. Eng.*, **123,** 809-812.

Lemos, M. L., Toranzo, A. E., and Barja, J. L. (1985) Antibiotic activity of epiphytic bacteria isolated from intertidal seaweeds. *Microbial. Ecol.*, **11,** 149-163.

Limbergen, H. V., Top, E., M., and Verstraete, W. (1998) Bioaugmentation in activated sludge: current features and future perspectives. *Appl. Microbiol. Biotechnol.*, **50,** 16-23.

Lindow, S. E., Panopoulos, N. J., and McFarland, B. L. (1989) Genetic engineering of bacteria from managed and natural habitats. *Science*, **244,** 1300-1306.

Lindqvist, R. and Bengtsson, G. (1991) Dispersal of dynamics of groundwater bacteria. *Microb. Ecol.*, **1,** 49-72.

Lotka, A. J. (1920) Elements of physical biology. Williams and Wilkins, Baltimore.

Lund, F. A. and Cabrera, M. M. (1986) Improved phenol oxidation in activated sludge by addition of phenol-adapted *Pseudomonas* sp. *Zentralbl. Mikrobiol.*, **141,** 115-119.

Macnaughton, S. J., Rose, D. A., and O'Donnell, A. G. (1992) Persistence of a *xylE* marker gene in *Pseudomonas putida* introduced into soils of differing texture. *J. General Microbiol.*, **138,** 667-673.

Mancini, P., Fertels, S., Nave, D., and Gealt, M. A. (1987) Mobilization of plasmid pHSV106 from *Escherichia coli* HB101 in a laboratory-scale waste treatment facility. *Appl. Environ. Microbiol.*, **53,** 665-671.

Matthess, G. and Pekdeger, G. (1981) Concept of a survival and transport model of pathogenic bacteria and viruses in groundwater. *Sci. Total. Environ.*, **21,** 149-159.

McClure, N. C., Weightman, A. W., and Fry, J. C. (1989) Survival of *Pseudomonas putida* UWC1 containing cloned catabolic genes in a model activated-sludge unit. *Appl. Environ. Microbiol.*, **55,** 2627-2634.

McClure, N. C., Fry, J. C., and Weightman, A. W. (1991) Survival and catabolic activity of natural and genetically engineered bacteria in a laboratory-scale activated-sludge unit. *Appl. Environ. Microbiol.*, **57,** 366-373.

McCormic, B. A., Klemm, P., Krogfelt, K. A., Burghoff, R., Pallesen, L., Lauz, D. C., and Cohen, P. S. (1993) *Escherichia coli* F-18 phase locked 'on' for expression of type 1 fimbriae is a poor colonizer of the streptomycin-treated mouse large intestine. *Microb. Pathogen.*, **14,** 33-43.

Mezrioui, N. and Baleux, B. (1994) Persistence patterns of *E. coli* strains isolated from domestic sewage before and after treatment in both aerobic lagoon and activated sludge. *Wat. Res.*, **28,** 2399-2406.

Mezrioui, N., Blaeux, B., and Troussellier, M. (1995) A microcosm study of the survival of *Escherichia coli* and *Salmonella typhimurium* in brackish water. *Wat. Res.*, **29,** 159-465.

Mohamed, A. R., Ossama, M. E., and Alexander., M. (1990) Inoculum sizes as a factor limiting success of inoculation for biodegradation. *Appl. Environ. Microbiol.*, **56,** 1392-1396.

Muyima, N. Y. O. and Cloete, T. E. (1995) Growth and phosphate uptake of immobilized *Acinetobacter* cells suspended in activated sludge mixed liquor. *Wat. Res.*, **29,** 2461-2466.

Nair, S. and Simidu, U. (1987) Distribution and significance of heterotrophic marine bacteria with antibacterial activity. *Appl. Environ. Microbiol.*, **53,** 2957-2962.

Nüblein, K., Maris, D., Timmis, K., and Dwyer, D. F. (1992) Expression and transfer of engineered catabolic pathways harbored by *Pseudomonas* spp. introduced into activated sludge microcosms. *Appl. Environ. Microbiol.*, **58,** 3380-3386.

Okada, M., Nakamura, T., Ito, H., and Murakami, A. (1991) Population dynamics of phenol degrading microorganisms in activated sludge process under the stress of phenolic wastewater. *Wat. Sci. Tech.*, **23,** 1001-1010.

Peterson, T. C. and Ward, R. C. (1989) Development of a bacterial transport model for coarse soils. *Wat. Resour. Bulltein.*, **25,** 349-357.

Pike, E. B., Carrington, E. G., and Ashburner, P. A. (1972) An evaluation of procedures for enumerating bacteria in activated sludge. *J. Appl. Bacteriol.*, **35,** 309-321.

Pickup, R. W. , Morgan, J. A. W., Winstanley, C., and Saunders, J. R. (1991) Implications for the release of genetically engineered organisms. *J. Appl. Bacteriol. Symp. Suppl.*, **70,** 19-30.

Poter, M. C. (1990) Handbook of industrial membrane technology. Noyes, USA.

Ramos, J. L., Diaz, E., Dowling, D., de Lorenzo, V., Molin, S., O'Gara, F., Ramos, C., and Timmis, K. N. (1994) The behavior of bacteria designed for biodegradation. *Bio/Technol.*, **12,** 1349-1356.

Recorbet, G., Steinberg, C. and Faurie, G. (1992) Survival in soil of genetically engineered *Escherichia coli* as related to inoculum density, predation and competition. FEMS *Microbiol. Ecol.*, **101,** 251-260.

Recorbet, G., Picard, C., Nomand, P., and Simonent, P. (1993) Kinetics of the persistence of chromosomal DNA from genetically engineered *Escherichia coli* introduced into soil. *Appl. Environ. Microbiol.*, **59,** 4289-4294.

Rittmann, B. E. (1982) Comparative performance of biofilm reactor types. *Biotechnol. Bioeng.*, **24,** 1341-170.

Rittmann, B. E. (1992) Development and experimental evaluation of a steady-state, multispecies biofilm model. *Biotechnol. Bioeng.*, **39,** 914-922.

Rosenberg, M., Gutnick, D., and Rosenberg, E. (1980) Adherence of bacteria to hydrocarbons: A simple method for measuring cell-surface hydrophobicity. *FEMS Microbiol. Lett.*, **9,** 29-33.

Rosenfeld, W. D. and Zobell, C. E. (1947) Anitibiotic production by marine microorganisms. *J. Bacteriol.*, **54,** 393-398.

Roszak, D. B. and Colwell, R. R. (1987) Survival strategies of bacteria in the natural environment. *Microbiol. Rev.*, **51,** 365-379.

Sakai, Y., Mori, T., Iida, M., Honda, K., and Matsumoto, T. (1982) Scum formation by acinetomycetes (*Norcadia* sp.) in final clarifer of activated sludge process. *J. Japan Sew. Wks. Assoc.*, **19,** 56-65. (in Japanese)

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular cloning, a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, New York.

Selvaratnam, S., Schoedel, B. A., McFarland, B. L., and Kulpa, C. F. (1997) Application of the polymerase chain reaction (PCR) and reverse transcriptase/PCR for determining the fate of phenol-degrading *Pseudomonas putida* ATCC 11172 in a bioaugmented sequencing batch reactor. *Appl. Microbiol. Biotechnol.*, **47,** 236-240.

Shimizu, N., and Odawara, Y. (1985) Floc-forming bacteria isolated from activated sludge in high-BOD loading treatment. *J. Ferment. Technol.*, **63,** 67-71.

Shohan, Y. and Demain, A. L. (1991) Kinetics of loss of recombinant plasmid in *Bacillus subtilus*. *Biotech. Bioeng.*, **37,** 927-935.

Short, K. A., Doyle, J. D., King, R. J., Seidler, R. J., Stotzky, G., and Olsen, R. H. (1991) Effects of 2,4,-dichlorophenol, a metabolite of a genetically engineered bacterium, and 2,4-dichlorophenoxyacetate on some microorganisms-mediated ecological processes in soil. *Appl. Environ. Microbiol.*, **57,** 412-418.

Simkins, S. and Alexander, M. (1984) Models for mineralization kinetics with the variables of substrate concentration and population density. *Appl. Environ. Microbiol.*, **47,** 1299-1306.

Simonsen, L. (1991) The existence conditions for bacterial plasmids: Theory and reality. *Microb. Ecol.*, **22,** 187-205.

Sinclair, J. L. and Alexander, M. (1984) Role of resistance to starvation in bacterial survival in sewage and lake water. *Appl. Environ. Microbiol.*, **48,** 410-415.

Sinclair, J. L. and Alexander, M. (1989) Effect of protozoan predation on relative abundance of fast- and slow-growing bacteria. *Can. J. Microbiol.*, **35,** 578-582.

Snape, J. B., Dunn, I. J., Ingham, J., and Prenosil, J. E. (1995) Dynamics of environmental bioprocess. Modeling and simulation. VCH Publishers, New York.

Stephenson, D. and Stephenson, T. (1992) Bioaugmentation for enhancing biological wastewater treatment. *Biotech. Adv.*, **10,** 549-559.

Sudhani, U. V., Bhatt, N. M., and Singh, M. (1991) Phenol toxicity on the biomass from an activated sludge plant treating petrochemical effluent. *Indian J. Environ. HLTH*, **33,** 306-311.

Sumino, T., Nakamura, H., Mori, N., and Kawaguchi, Y. (1992) Immobilization of nitrifying bacteria by polyethylene glycol prepolymer. *J. Ferment. Bioeng.*, 73, 37-42.

Tago, Y., Kuraishi, H., and Aida, K. (1975) The formation of a model floc able to decompose phenol by the mixed culture of bacteria isolated from activated sludge. J. *Gen. Appl. Microbiol.*, **21,** 41-49.

Takeo, M., Maeda, Y., Okada, H., Miyama, K., Mori, K., Ike, M., and Fujita, M. (1995) Molecular cloning and sequencing of the phenol hydroxylase gene from *Pseudomonas putida* BH. *J. Ferment. Bioeng.*, **79,** 485-488.

Tanaka, H., Kurano, N., Ueda, S., Okazaki, M., and Miura, Y. (1985) Model system of bulking and flocculation in mixed culture of *Sphaerotilus* sp. and *Pseudomonas* sp. for dissolved oxygen deficiency and high loading. *Wat. Res.*, **19,** 563-571.

Tanaka, N., Inamori, Y., Kawabata, Z., Mori, T., Itayama, T., and Sudo, R. (1995) Simulation of the population dynamics of a genetically engineered microorganism in a microcosm. *Japanese J. Wat. Biol.*, **31,** 33-41. (in Japanese)

Tang, Y. J. and Alexander, M. (1987) Absence of a role for lytic microorganisms in decline of bacteria and *Saccharomyces* introduced into soil. *Microbial. Ecol.*, **14,** 67-73.

Tiedje, J. M., Colwell, R. K., Grossman, Y. L., Hodson, R. E., Lenski, R. E., Mack, R. N., and Regal, P. J. (1989) The planned introduction of genetically engineered organisms: ecological considerations and recommendations. *Ecology*, **70,** 298-315.

Timmis, K. N. (1997) Design of bacteria for environmental applications. In: Verachtert, H. and Verstracte, W. (edn) Proceedings of the international symposium on environmental biotechnology (ISEB) Technological Institute, Ostend, 1997. pp 11-22.

Top, E., Mergeay, M., Springael, D., and Vestraete, W. (1990) Gene escape model: Transfer of heavy metal resistance genes from *Escherichia coli* to *Alcaligenes eutrophus* on agar plates and in soil samples. *Appl. Environ. Microbiol.*, **56,** 2471-2479.

Travis, C. C. and Etnier, E. L. (1981) A survey of sorption relationships for reactive solutes in soil. *J. Environ. Qual.*, **10,** 8-17.

Tyagi, R. D. , Du, Y. G., and Bhamidimarri, R. (1996) Dynamic behavior of the activated sludge process under shock loading: application of the floc model. *Wat. Res.*, **30,** 1605-1616.

Unz, R. F. and Dondero, N. C. (1967) The predominant bacteria in natural Zoogloeal colonies. II. Physiology and nutrition. *Can. J. Microbiol.*, **13,** 1683-1694.

van der Drift, D., van Seggelen, E., Stumm, C., Hol, W., and Tuinte, J. (1977) Removal of *Escherichia coli* in wastewater by activated sludge. *Appl. Environ. Microbiol.*, **34,** 315-319.

Verstraete, W. and van Vaerenberg, E. (1986) Aerobic activated sludge. In: Rehm, H. J. and Reed, G. (eds) Biotechnology. vol. 8. VCH. Weinheim, pp 44-102.

Vilker, V. L. (1980) Adsorption mass transfer model for virus transport in soils. *Wat. Res.*, **30,** 783-790.

Wand, H., Laht, T., Peters, M., Becker, P. M., Stottmeister, U., and Heinaru, A. (1997) Monitoring of biodegradative *Pseudomonas putida* strains in aquatic environments using molecular techniques. *Microb. Ecol.*, **33,** 124-133.

Wang, J. G. and Bakken, L. R. (1998) Screening of soil bacteria for poly-β-hydroxybutyric acid production and its role in the survival of starvation. *Microbial Ecol.*, **35,** 94-101.

Wanner, O. and Gujer, W. (1986) A multispecies biofilm model. *Biotechnol. Bioeng.*, **28,** 314-328.

Watanabe, K. and Hino, S. (1996) Identification of a functional important population in phenol-digesting activated sludge with antisera raised against isolated bacterial strains. *Appl. Environ. Microbiol.*, **62,** 3901-3904.

Watanabe, K., Hino, S., and Takahashi, N. (1996) Effects of exdigenous phenol-degrading bacteria on performance and ecosystem of activated sludge. *J. Ferment. Bioeng.*, **82,** 291-298.

Watanabe, K., Yamamoto, S., Hino, S., and Harayama, S. (1998) Population dynamics of phenol-degrading bacteria in activated sludge determined by *gyrB*-targeted quantitative PCR. *Appl. Environ. Microbiol.*, **64,** 1203-1209.

Wiggins, B. A. and Alexander, M. (1988) Role of chemical concentration and second carbon sources in acclimation of microbial communities for biodegradation. *Appl. Environ. Microbiol.*, **54,** 2803-2807.

Wilder, P. A., Rubio, M. A., and Davids, L. (1991) Impact of addition of pure cultures on the performance of mixed culture reactors. *Wat. Res.*, **25,** 1307-1313.

Zaidi, B. R., Murakami, Y., and Alexander, M. (1989) Predation and inhibitors in lake water affect the success of inoculation to enhance biodegradation of organic chemicals. *Environ. Sci. Technol.*, **23,** 859-863.

Zita, A. and Hermansson, M. (1997) Effects of bacterial cell surface structure and hydrophobicity on attachment to activated sludge flocs. *Appl. Environ. Microbiol.*, **63,** 1168-1170.

Publications Related to This Study

Soda, S., Ike, M., and Fujita, M. (1998) Effects of inoculation of a genetically engineered bacterium on performance and indigenous bacteria of a sequencing batch activated sludge process treating phenol. *J. Ferment. Bioeng.*, **86,** 90-96.

Soda, S., Watatani, H., Ike, M., and Fujita, M. (1998) Factors affecting the survival of exogenous bacteria in microbial ecosystems; existence of indigenous bacteria with antagonistic activity. *Biocontrol Sci.*, **3,** 63-72.

Fujita, M., Ike, M., Nakamura, F., and Soda, S. (1998) Isolation and characterization of a floc-forming bacterium *Sphingomonas paucimobilis* 551 from activated sludge. *Japanese J. Wat. Tret. Biol.*, **34,** 195-205.

Soda, S., Ike, M., and Fujita, M. (1999) Adsorption of bacterial cells onto activated sludge. *J. Biosci. Bioeng.*, **87,** (in press).

Soda, S., Ike, M., and Fujita, M. (1999) Simulation study of competition between two microorganisms with antagonistic relationships in a completely mixed reactor. *Biocontrol Sci.*, **4,** (in press).

Soda, S., Heinzle, E., and Fujita, M. Modeling and simulation of competition between two microorganisms for a single inhibitory substrate in a biofilm reactor. *Biotecnol. Bioeng.* (Submitted).

Soda, S., Uesugi, K., Ike, M., and Fujita, M. Application of a floc-forming genetically engineered microorganism to a sequencing batch reactor for phenolic wastewater treatment. *J. Biosci. Bioeng.*, (Submitted).