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Fate in sewage of a recombinant *Escherichia coli* K-12 strain used in the commercial production of bovine somatotropin

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SUMMARY

The fate of a derivative of *Escherichia coli* strain W3110G [pBGH1], a strain used for production of bovine somatotropin, was examined in semi-continuous activated sludge (SCAS) units. A nalidixic acid-resistant derivative of W3110G [pBGH1], strain LBB270 [pBGH1], was used to facilitate tracking. SCAS units (300 ml) containing municipal mixed liquor were operated on a daily cycle of 23 h aeration and 1 h settling followed by decanting of clear supernatant (175 ml) and refilling with fresh primary effluent. SCAS units were inoculated with two concentrations of *E. coli* LBB270 [pBGH1] and operated for 200 h. Viable levels of *E. coli* LBB270 [pBGH1] were measured daily in aerated mixed liquor and decanted supernatant. Viable counts in the mixed liquor decreased from 10000- to 100000-fold in less than 200 h. Losses of *E. coli* LBB270 [pBGH1] in decanted supernatants accounted for less than 2-fold of the total losses observed in the SCAS units. The *E. coli* LBB270 [pBGH1] was not evenly distributed in the mixed liquor, but became preferentially associated with the settleable floc. These results show that *E. coli* LBB270 [pBGH1] was unable to survive in municipal sludge even when inoculated at concentrations greater than, or comparable to, levels of indigenous microorganisms.

INTRODUCTION

Safety interests have been an essential part of the discovery and development of new biotechnology-based processes. Early concerns for human health led to the initial selection and subsequent widespread use of *Escherichia coli* K-12, a debilitated strain known to be incapable of colonizing the mammalian intestinal tract [10–13,16], as the safest microbial host for recombinant systems. Although industrial fermentations using *E. coli* K-12 may reach cell densities greater than 1×10^9 cells per ml [4,6], these closed fermentation systems are highly contained and all viable cells in the process wastewater are killed prior to discharge. However, there has been concern for the possible environmental impact of an inadvertant release of high concentrations of recombinant *E. coli* K-12 to soil, water or domestic sewage plants.

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The environmental concerns for recombinant bacteria have centered on the potential for released microorganisms to survive in different ecosystems and transfer recombinant DNA to indigenous organisms. A recent extensive review of published studies investigating these issues [2] concluded that strains of *E. coli* K-12 do not persist in nonsterile water, soil, sewage or the conventional mammalian intestinal tract. Furthermore, this review concluded that the conjugational transfer of pBR322, or derivatives of pBR322, from strains of *E. coli* K-12 to indigenous inhabitants of water, soil or sewage in their natural environment has never been demonstrated [2]. Although these reviewed studies were not conducted with recombinant *E. coli* K-12 strains actually used for commercial production, the results demonstrate the safety of *E. coli* K-12 to humans and the environment.

Monsanto Company uses *E. coli* K-12 strain W3110G containing pBGH1, a plasmid derived from pBR322, for the commercial production of bovine somatotropin (BST) [4,6]. Although a number of systems are in operation to prevent the escape of this recombinant microorganism from fermentation areas, several studies have recently been conducted to determine the environmental fate of *E. coli* W3110G [pBGH1] outside of the production plant. It was recently reported that this commercial *E. coli* K-12 strain did not survive in environmental sources of water or ad-

versely affect populations of indigenous microorganisms in natural waters [1]. The present study utilized semi-continuous activated sludge (SCAS) units containing domestic activated sludge to model the fate of *E. coli* W3110G [pBGH1] in sewage.

MATERIALS AND METHODS

Media and reagents

Levine Eosin Methylene Blue (EMB) agar and plate count agar media were obtained from Difco Laboratories (Detroit, MI), and prepared according to the instructions of the supplier. For this study, the plate count agar was referred to as tryptone-yeast extract-glucose (TYG) agar. The antibiotic nalidixic acid (Sigma Chemical Co., St. Louis, MO) was added to EMB medium to a final concentration of 200 mg per liter. L-broth contained 10 g of tryptone, 5 g of yeast extract, 5 g of sodium chloride, and 1 g of glucose per liter [9]. L-broth agar plates were prepared from L-broth containing 15 g Bacto agar per liter. Bottles of sterile 0.1% peptone water were obtained from Fisher Scientific (Pittsburgh, PA).

Experimental design

This study used a total of eight semi-continuous activated sludge (SCAS) units; two uninoculated controls and six receiving *E. coli* LBB270 [pBGH1]. Assuming that the concentration of cells in a fermenter would be diluted, after an accidental discharge, from 100–1000-fold before reaching a sewage treatment plant, *E. coli* LBB270 [pBGH1] would be diluted from 3×10^9 cell per ml (a typical concentration for a fermentation sample) to approx. 3×10^6 to 3×10^7 cells per ml in the oxidation basins. These experiments were conducted with 10^5 to 10^7 *E. coli* LBB270 [pBGH1] cells per ml of mixed liquor. At these concentrations, the recombinant *E. coli* would be present at numbers greater than, or equal to, the indigenous populations. We chose this approach in order to favor the establishment of the *E. coli* LBB270 [pBGH1] in these SCAS units.

This experimental plan considered three possible sources of variation: variability among different fermenter batches, experimental variation and measurement variation. Fundamental methods of replication, blocking, controls and randomization were implemented to deal with those possible sources of variation [3,5]. This experiment was performed in two blocks. Each block contained three fermentation batches and negative controls. This block structure was achieved by repeating an entire trial at two different times using fresh materials (fermentation batches, primary effluent, sewage liquor, media, etc.). Repeating the trials provided protection against unintended variation due to random experimental variation related to procedure, such as addition of fresh primary effluent, collection of samples, etc. During each trial, each of three fermentation batches was inoculated at both a high and a low level into SCAS units. Two SCAS units were uninoculated to serve as negative controls. The eight SCAS units were assigned using a computer randomization routine. The two levels of inoculation were included to provide for the possibility that survival depended on the relative proportions of inoculated and indigenous microorganisms.

Collection and preparation of sewage

The mixed liquor (aerated activated sludge) and primary effluent (raw sewage influent) were collected from the Grand Glaize Wastewater Treatment Facility (St. Louis, MO). One week prior to the beginning of the study extraneous particulate matter was removed from both the mixed liquor and primary effluent by filtering these liquids through a 20-mesh stainless steel screen and glass wool, respectively. The mixed liquor was concentrated by settling and decanting of supernatant to a suspended solids content of 1000 to 2000 mg per liter. The mixed liquor was placed at 25 °C in a 10-liter reservoir where it was aerated, except for a daily maintenance period, until used. Maintenance consisted of settling the sludge solids, draining the supernatant, and recharging with the primary effluent collected at the same time as the mixed liquor. The primary effluents used in these experiments were stored at 2° to 6 °C, but were placed in a 25 °C incubator for 1 h before use.

Semi continuous activated sludge (SCAS) units

A schematic of the SCAS unit used in this study is shown in Fig. 1. Each unit was made of glass and held approx. 300 ml of liquid. There were ports on the sides of the unit which were used to decant supernatant and add fresh primary effluent. A stopcock on the bottom of the unit introduced a controlled flow of air to the mixed liquor. A hydrophobic filter was in the air line to prevent the backflow of material from the SCAS unit to the air manifold. An exhaust air line was connected from the screw top cap of the unit to a container of disinfectant, so that any microorganisms present in the exhaust air would be killed. The units were connected to a compressed air manifold, and the air flow was adjusted to 50 cc per min using a Gilian Flow Calibrator, connected to a bubble generator. The units were maintained at 24 °C to 26 °C in the dark. Lights were turned on only when the units were being sampled or cycled.

One day prior to the start of the experiment, 300 ml of mixed liquor were added to each of the units. The level of liquid in each unit was marked on the glass, and air flow was initiated by opening the stopcock. The bubbling served to mix the contents of the SCAS unit as well as aerate the mixed liquor. After 24 h, aeration was stopped, and the

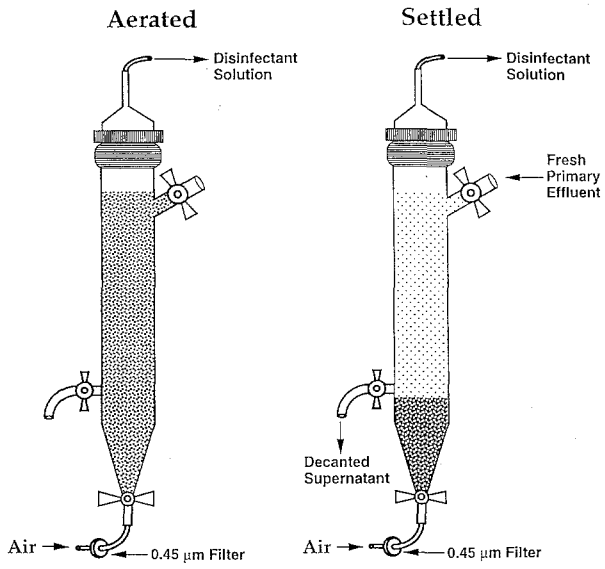

Fig. 1. Schematic of the semi-continuous activated sludge (SCAS) reactor system used in this study.

level of liquid in the unit was determined. If the liquid was below the 300 ml mark, sterile deionized water was added to bring the volume back to the starting point, and aeration was started. Air was bubbled through the units for 5 min to mix the contents. Aeration was again stopped, and the sludge was allowed to settle for 1 h. From each of the SCAS units 175 ml of supernatant was removed, and each unit was refilled with fresh primary effluent; at this time the appropriate units were inoculated with recombinant *E. coli*.

Isolation of a nalidixic acid resistant E. coli *strain*

E. coli strain W3110G [pBGH1] was marked with a chromosomal mutation that resulted in resistance to the antibiotic nalidixic acid in order to facilitate tracking of the recombinant microorganism among indigenous sewage microbes. The properties of a nalidixic acid-resistant *E. coli* have been well described, and such mutations have been found to occur at the genetic locus *gyrA* [15]. A spontaneous mutation to nalidixic acid resistance was obtained in the *E. coli* host as follows. Strain W3110G [pBGH1] was inoculated onto the surface of a Luria agar plate containing 200 μg of nalidixic acid per ml of medium. This concentration of the antibiotic inhibits growth and allows for the selection of nalidixic acid resistant derivatives. The plates were incubated at 37 °C until resistant colonies appeared. Several such colonies were streaked onto Luria agar with nalidixic acid. All appeared to have the same colony morphology and size, so one colony was selected and designated LBB270 [pBGH1]. In addition to nalidixic acid resistance, *E. coli* strain LBB270 [pBGH1] was also resistant to tetracycline and ampicillin by virtue of harboring the plasmid pBGH1. Including all three of these antibiotics in the EMB agar reduced the number of indigenous microbes by a factor of 10^4 to 10^6. Thus, the use of *E. coli* LBB270 [pBGH1] allowed plating of a 0.1-ml aliquot of the undiluted sewage samples directly onto the EMB antibiotic plates, giving a limit of detection of approximately 10 cells per ml.

Fermentation of recombinant E. coli

E. coli strain LBB270 [pBGH1] was grown in 15-l Biolafitte fermenters. The temperature was maintained at 37 °C until an optical density at 550 nm of 20 to 25, at which point the temperature was decreased to 30 °C. At an optical density of 40 to 45, indole acrylic acid was added to a final concentration of 25 ppm to initiate high level synthesis of BST. The cultures were maintained in the fermentation vessels for 10 h after the addition of indole acrylic acid at which point a 50-ml aliquot from each of the six independent fermentations was removed, placed in a sterile 50-ml polypropylene tube, and maintained at 2 °C to 6 °C until needed. Separate fermentations were conducted for each experimental trial.

Inoculation and operation of SCAS units

We ran two trials of this experiment. In each case, eight SCAS units were maintained for approx. 200 h. Three SCAS units received a high inoculum (approx. 1.0×10^7 cells per ml), three SCAS units received a low inoculum (approx. 1.0×10^5 cell per ml), and two SCAS units were uninoculated controls. The high inocula were taken directly from the 50 ml fermentation samples. The low inocula were made by diluting the fermentation samples 100-fold into 0.1% peptone water. Three-milliliter aliquots of *E. coli* LBB270 [pBGH1] from either the high or low inocula samples were added to the SCAS units that had just received the 175 ml of primary effluent. This represented an additional 100-fold dilution of the *E. coli* LBB270 [pBGH1]. Thus, the high inocula were diluted a total of about 100-fold, and the low inocula were diluted a total of about 10000-fold.

Aeration was begun immediately after inoculation for 5 to 10 min to allow the sludge and primary effluent to mix and a 2-ml sample was removed. This sample was designated 0 time. In trial 1, 2-ml samples were removed at 3, 6, and 23 h after inoculation. In trial 2, a sample was not taken at hours 3 and 6 but was taken at hour 23. The 2-ml samples from hours 3 and 6 were removed, while the unit was aerated in order to determine the viable counts in the mixed liquor. Prior to sampling at hour 23, aeration was turned off, and sterile deionized water was added to bring the volume back to 300 ml. Aeration was resumed to obtain a uniform mixture. After a 5–10-min period, a 2-ml

sample was taken, the air was turned off, and the sludge was allowed to settle. A 175-ml volume of supernatant was removed and replaced with fresh primary effluent. The flow of air was started and maintained for at least 5 but not more than 10 min to mix the contents of the SCAS unit. At this point, a 2-ml sample of the mixed liquor was obtained. Viable counts were determined in the mixed liquor, the supernatant, and the fresh primary effluent which was added to the units. This procedure for operating and sampling the SCAS units represented the first daily cycle. Subsequent daily cycles were similar, but did not include inoculation with recombinant *E. coli*. The units were operated for 200 h.

Two units were not inoculated with *E. coli* LBB270 [pBGH1] but were treated in a similar fashion. These units were monitored with dissolved oxygen probes on a daily basis to measure changes in dissolved oxygen levels. A drop in the dissolved oxygen concentration was taken as evidence of biological activity. These units were also sampled as described above. We opted not to use the dissolved oxygen probe on the inoculated units in order to avoid cross contamination of the inoculated and uninoculated units since it would be difficult to sterilize the probe between samples.

Enumeration of bacteria

Eosin methylene blue (EMB) agar plates and tryptone yeast glucose (TYG) agar plates were prepared as described by the supplier. EMB plates contained the antibiotics tetracycline, ampicillin and nalidixic acid at final concentrations of 10, 100 and 200 mg per liter, respectively.

All experimental samples were appropriately diluted to achieve microbial spread plate counts within the range of 30–300 per plate. EMB plates were incubated at 37–38 °C for 22 to 26 h. TYG plates were incubated at 26–28 °C for 45 to 51 h. Colonies were counted using a New Brunswick manual colony counter equipped with a light and electronic counting probe. Although we targeted the dilutions to achieve microbial plate counts in the 30–300 range, plates containing numbers outside this range were not discarded, but were included in the maximum likelihood estimation procedure [7], since we considered that every plate provided useful information. The Poisson and binomial models for number of bacteria both lead to the same estimate. Whenever possible we used a method described by Koch [8]; that is, 'to get the best estimate from a group of plates from the same or different dilutions of the same sample, simply add up the total counts on all the plates and divide it by the total volume of the original solution'. When the least dilute plate yielded greater than 300 colonies and actual reported counts were available from the other plates, a numerical optimization program provided the maximum likelihood estimate.

Statistical analyses

Bacterial plate counts were examined for consistency with counts at other dilutions from the same SCAS unit sample using a Chi^2 statistic. A mean viable count was determined for each inoculation condition ad the geometric mean of the corresponding results. This was achieved by taking the antilog of the arithmetic mean of the logarithms of the viable count estimates. This method is appropriate for a variable which is typically measured or reported on a logarithmic scale [14], and aids in stabilization of variance.

RESULTS AND DISCUSSION

Microbial populations in primary effluents

The total aerobic microbial counts of primary effluents for experimental trials 1 and 2 at 0 time were 1.8×10^6 to 1.3×10^6 cells per ml, respectively. The viable counts on EMB antibiotic plates for these same samples were 1.6×10^3 and 2.1×10^2, or 10^3–10^4-fold less than the total number of aerobic cells. The microbial population of both primary effluents remained essentially constant for the duration of the experiment. The total aerobic counts for a second collection of primary effluent were 9.5×10^5 to 1.8×10^6 cells per ml for trial 2. These results show that primary effluents collected approx. 3 weeks apart contained similar microbial counts and storage of these effluents at 2–6 °C had no adverse effects on the microbial populations.

In preliminary studies, samples of the primary effluent were plated on EMB antibiotic plates in order to determine the relative populations of antibiotic resistant lactose positive microorganisms. None were found in these tests of the primary effluents. However, in some of the subsequent analyses we did observe lactose positive colonies that were resistant to the three antibiotics. These microorganisms were identified as *Enterobacter cloacae* and *Citrobacter freundii*. No *E. coli* were identified in any of these tests of antibiotic-resistant indigenous microbes. A number of colonies taken from TYG plates containing samples of the primary effluent were also identified, and included *Acinetobacter calcoaceticus, Aeromonas hydrophila, Klebsiella pneumoniae, Flavobacterium* sp., and some unidentified non-fermenting Gram-negative bacteria.

Microbial populations in uninoculated SCAS units

The total aerobic counts in the uninoculated SCAS units are shown in Fig. 2. At the start of each cycle (that is, after primary effluent was added to the units) the viable counts were from 9.6×10^5 to 3.6×10^6 cells per ml. At the end of each cycle these counts dropped to 3.2–4.7×10^5 cells per ml. These data imply that there is some decrease

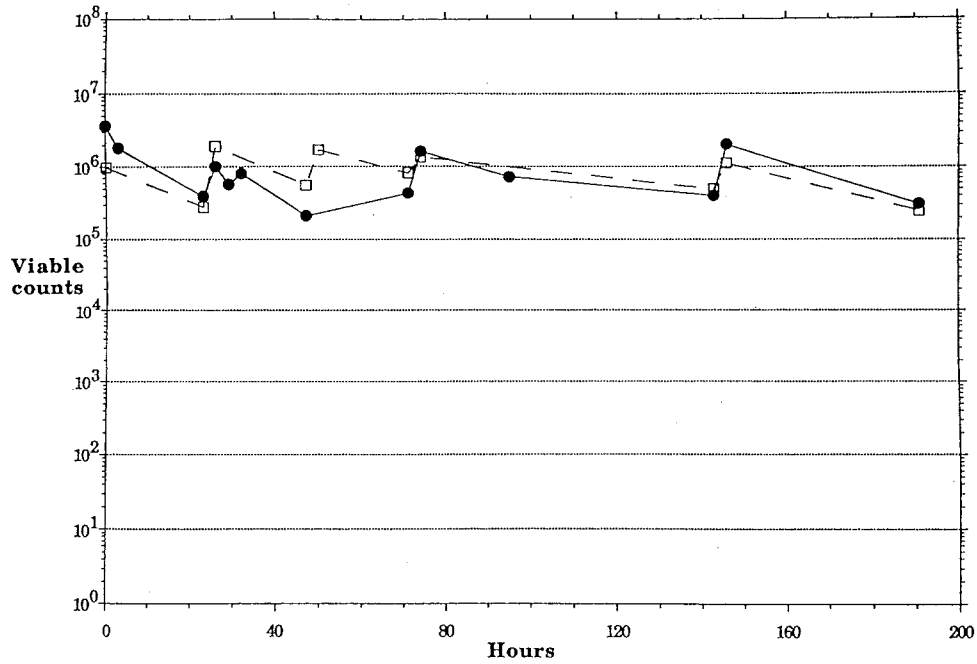

Fig. 2. Mean total aerobic microbial counts in SCAS units 1 and 2 with no *E. coli* LBB270 [pBGH1] addition. Microbial counts are shown for experimental trial 1 (–●–) and trial 2 (– –□– –).

in the indigenous populations during the 24 h residence time. Since the respiratory rates declined after approx. 6 h, this may indicate that there is not enough carbon to maintain a microbial population much in excess of $3–5 \times 10^5$ cells per ml.

Inoculation of SCAS units

The undiluted 50-ml fermentation samples from each fermentation were the source of the high inocula of *E. coli* LBB270 [pBGH1]. The fermentation sample from the fermentation used in the first experimental trial contained $1.5–1.7 \times 10^9$ cells per ml. The fermentation samples for the second trial contained $4.3–9.6 \times 10^8$ cells per ml. The source of low inocula (the 100-fold dilution of the fermentation samples) for the first and second trials contained $1.3–1.6 \times 10^7$ cells/ml and $2.9–6.1 \times 10^6$ cells/ml, respectively. Three mls of high or low inoculum were added to the designated SCAS units to begin the study. The number of *E. coli* LBB270 [pBGH1] in SCAS units 3, 5, and 7 (which had received the high inocula) was $1.0–5.0 \times 10^7$ cells per ml, which was about 10-fold higher than the number of indigenous microorganisms. In SCAS units 4, 6, and 8, which had received the low inocula of *E. coli* LBB270 [pBGH1], the viable cell count was $1.0–5.0 \times 10^5$ or approx. 10-fold less than the indigenous microbial population.

Performance of the SCAS units

Uninoculated SCAS units 1 and 2 exhibited a daily pattern of oxygen use indicative of high biological activity. Although dissolved oxygen levels were normally near saturation prior to settling and decanting of supernatant, levels of dissolved oxygen characteristically dropped to 1.4–1.9 mg per liter within 2 h after the addition of fresh primary effluent. The units obtained dissolved oxygen levels of 4.6–6.0 mg per liter within 6 h and reached oxygen saturation prior to the next draining and feeding cycle (hour 23 of the daily cycle). This pattern was repeated every time fresh primary effluent was added to the units. Since the primary effluents contained approx. 80 ppm and 45 ppm of total organic carbon for trials 1 and 2, respectively, we propose that the microorganisms utilized this carbon source through an oxygen dependent respiratory pathway, and this was reflected in a decrease in the dissolved oxygen concentration.

Under normal operating conditions at the Grand Glaize sewage treatment plant, there is a continual influx of primary effluent (raw sewage containing nutrients and microorganisms) into the aeration basin containing the mixed liquor. The estimated holding time in this basin is approx. 5 h. We wanted to provide for this influx of fresh primary effluent, but we also wanted to increase the residence time for *E. coli* LBB270 [pBGH1] in the mixed li-

quor in order to improve the chances of establishing itself in the activated sludge. Since we were adding fresh primary effluent every 24 h; there was a possibility that the loss of viable *E. coli* LBB270 [pBGH1] was the result of washing out the cells during the draining and filling operation. Indeed, the supernatants of units 3, 5, and 7 containing the high inocula were turbid at hour 24, the first time the units were drained. This was not completely unexpected since the optical density at 550 nm of the 50-ml undiluted fermentation sample was approx. 100. Since this sample was diluted 100-fold, one would expect that the optical density of the supernatant would be approx. 1 if all of the cells were present in the supernatant. We determined the levels of *E. coli* LBB270 [pBGH1] in the supernatants and compared these values to the cell numbers in the samples taken just prior to turning off the aeration. These results, which are presented in Table 1, illustrate the percentage of viable *E. coli* LBB270 [pBGH1] found in the drained supernatants. At the first change of primary effluent at hour 24 there were between 3.9% and 22.6% of the inoculated cells in the drained supernatant fractions. At subsequent transfer times, however, there was 3% or less of *E. coli* LBB270 [pBGH1] in these drained supernatants. Altogether, there was less than a 2-fold loss of *E. coli* LBB270 [pBGH1] as a result of draining and filling, whereas the viable cell count in the mixed liquor had dropped 10000–100000-fold during the course of the experiment. These results demonstrate that the major loss of *E. coli* LBB270 [pBGH1] in these SCAS units could not be accounted for by washing out during the draining and filling operations. Furthermore, there appeared to be an uneven distribution of *E. coli* LBB270 [pBGH1] in these units. The recombinant *E. coli* appeared to be primarily associated with the sludge and not the supernatant.

TABLE 1

Percentage of *E. coli* LBB270 [pBGH1] cells removed in decanted supernatant from inoculated semi-continuous activated sludge units[a]

	Removal time (h)	SCAS 3[b] (%)	SCAS 5[b] (%)	SCAS 7[b] (%)
Trial 1	24	13.8	3.9	11.1
	48	0.6	0.1	3.0
	72	0.3	0.4	1.9
	96	0.1	0.1	0.1
	144	0.4	0.3	0.2
Trial 2	24	16.3	22.6	21.0
	48	1.3	1.2	1.7
	72	0.6	0.8	2.3
	96	<0.7	<0.8	0.1

[a] The total *E. coli* LBB270 [pBGH1] cells in decanted supernatant are expressed as a percentage of the total *E. coli* LBB270 [pBGH1] population in the SCAS unit 1 h before each settling and decanting of supernatant.

[b] These units were inoculated with the high concentration of recombinant *E. coli*.

Similar results were observed with the supernatants from the units containing the low inocula. Ratios could not be determined since most of the supernatant had viable counts below the limit of detection. Yet the viable counts of *E. coli* LBB270 [pBGH1] continually decreased in the mixed liquor in these units as well.

Effect of high inocula on SCAS units

The inocula of *E. coli* LBB270 [pBGH1] altered the mixed liquor of the SCAS units as evidenced by turbidity and a drop in pH (Fig. 3) of the supernatant fractions. The turbidity was not surprising given the high level of inoculation, and pH changes were not unexpected since the fermentation broth contained 10000 mg per liter of acetate and 1000 mg per liter of glucose. The addition of 3 mls of this broth to 300 ml of mixed liquor would yield 100 and 10 mg per liter, respectively, of acetate and glucose in the SCAS units. Since the total organic carbon in the primary effluents was 80 ppm (trial 1) and 45 ppm (trial 2), these concentrations of organic carbon in the high inocula would be significant additions to the SCAS units.

Loss of high levels of E. coli *LBB270 [pBGH1]*

The microbial counts in SCAS units inoculated with a high level of *E. coli* LBB270 [pBGH1] are shown in Fig. 4. The *E. coli* LBB270 [pBGH1] was the major microorganism in the inoculated units at time 0. The indigenous viable counts from the uninoculated SCAS units were approx. $0.96–3.6 \times 10^6$ (Fig. 2), whereas the viable counts of *E. coli* LBB270 [pBGH1] on EMB antibiotic plates were $0.86–1.6 \times 10^7$ cells per ml. These results suggest that there were approx. 10-fold more *E. coli* than indigenous microorganisms in SCAS units 3, 5, and 7. The viable counts on TYG plates from SCAS units 3, 5, and 7 were $3.9–5.7 \times 10^7$ cells per ml.

On both types of plates, the viable counts of *E. coli* LBB270 [pBGH1] remained essentially constant for the first 24 h, after which they decreased. For the first 48 to 72 h (Fig. 4) the viable counts on EMB antibiotic plates were approx. 4-fold less than those seen on TYG plates. As the counts on EMB antibiotic plates dropped below 1.0×10^6, the counts on TYG remained around 1.0×10^6, which was the level of indigenous microbial populations (Fig. 2). We conclude from these results that the selective medium we used to detect LBB270 [pBGH1] gave an accurate reflection of its viability, and indicated that our plating media did not bias our results.

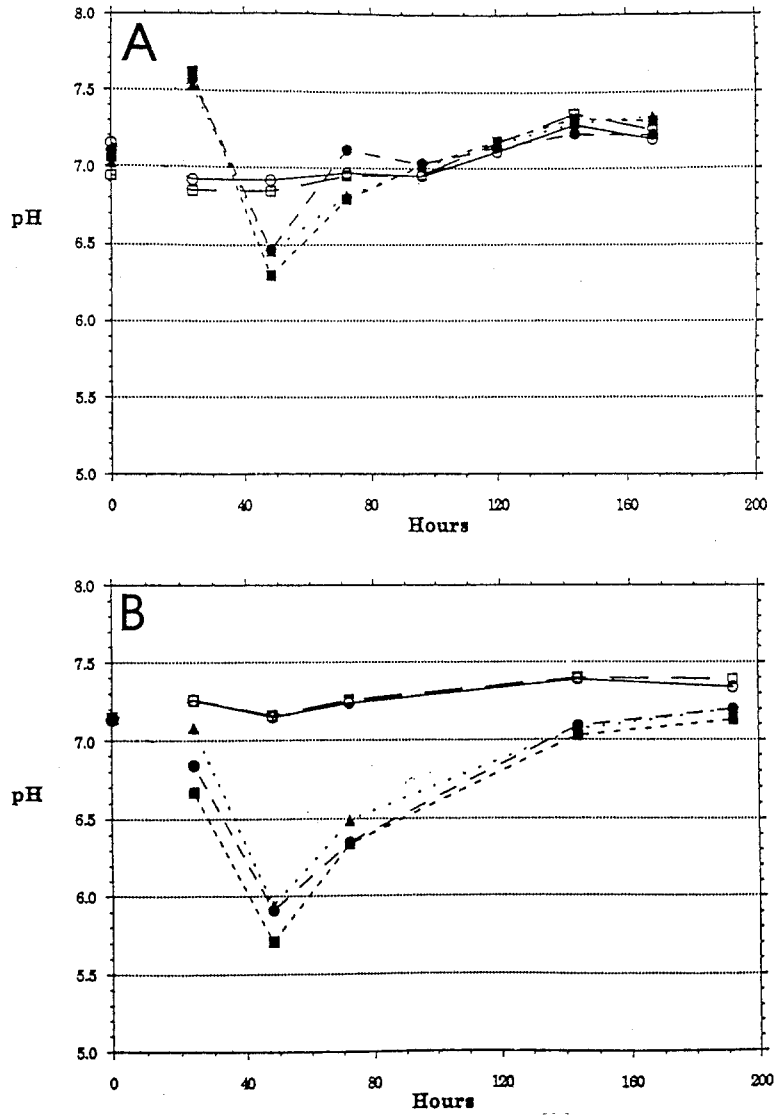

Fig. 3. The pH of drained supernatant fractions from experimental trials 1 (panel A) and 2 (panel B). SCAS units 1 (–○–) and 2 (– –□– –) were uninoculated controls and SCAS units 3 (– –●– –), 5 (– –■– –) and 7 (· ·▲· ·) were inoculated with a high concentration of *E. coli* strain LBB270 [pBGH1]. Since the pH at hour 0 was determined prior to inoculation, hour 24 represents the first pH determination for the inoculated SCAS units.

The viable count of *E. coli* LBB270 [pBGH1] in the mixed liquor dropped 10000–100000-fold over the course of this experiment. Although it is not clear why the viable counts of *E. coli* LBB270 [pBGH1] remained essentially constant for the first 24 h in these SCAS units, it is probably a reflection of the effect of adding such a high concentration of fermentation broth to these units. While such high concentrations of organic carbon (acetate and glucose) from the fermentation broth would be unlikely in an activated sludge oxidation basis, these results demonstrate that LBB270 [pBGH1] would not survive even under these optimal conditions.

Loss of low levels of E. coli *LBB270 [pBGH1]*

The microbial counts in SCAS units inoculated with a low level of *E. coli* LBB270 [pBGH1] are shown in Fig. 5. The low inocula produced no visible turbidity or significant changes in pH. Since an accidental release of *E. coli* would likely be diluted prior to reaching a sewage treatment plant, the low inocula probably represents a more realistic model to examine to potential for *E. coli* LBB270[BGH1] to establish itself in a municipal sewage treatment plant.

The viable counts of *E. coli* LBB270 [pBGH1] on EMB antibiotic plates decreased rapidly in these units from time

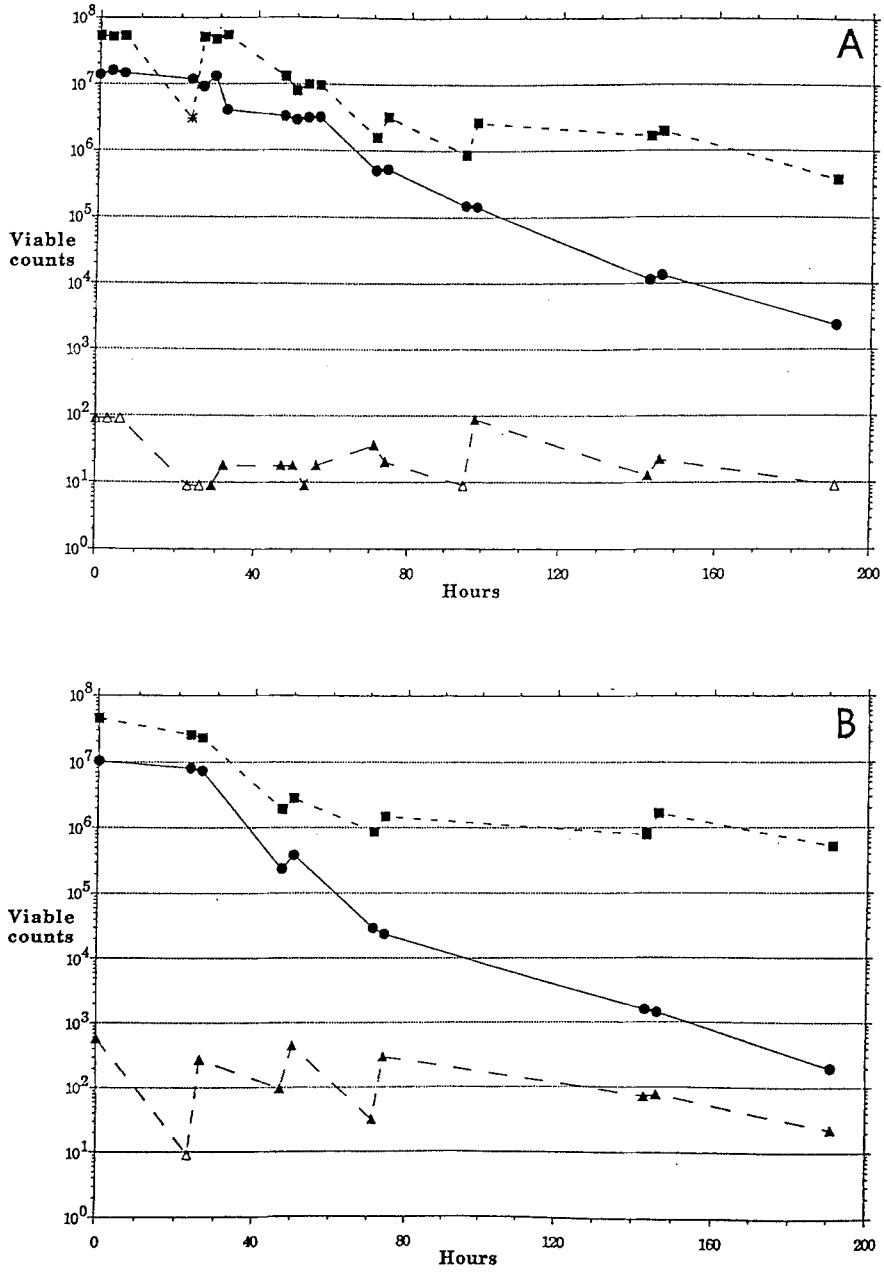

Fig. 4. Microbial counts for experimental trials 1 (panel A) and 2 (panel B) of SCAS units 3, 5, and 7 inoculated with a high concentration of *E. coli* strain LBB270 [pBGH1] and uninoculated SCAS units 1 and 2. The mean total aerobic microbial counts from SCAS units 3, 5, and 7 (– –■– –) were determined on TYG plates and includes *E. coli* strain LBB270 [pBGH1] and indigenous populations. The population of *E. coli* LBB270 [pBGH1] in SCAS units 3, 5, and 7 (–●–) and indigenous antibiotic-resistant bacteria in SCAS units 1 and 2 (– –▲– –) were determined on EMB antibiotic plates. Some values were greater than the value shown (– –* – –) because there were more than 300 colonies on these plates. Open symbols (– –△– –) indicate that the lowest dilution had no colonies indicating the actual microbial count was lower than these estimates.

0, and followed the same pattern in both trials. The viable counts fell greater than 1000-fold during the course of the experiment. The only difference between these two trials appeared to be in the level of antibiotic resistant cells in the uninoculated controls, and probably represents the variability seen in the microbial content of the primary effluents. These results show that when *E. coli* LBB270 [pBGH1] was inoculated into these SCAS units at a con-

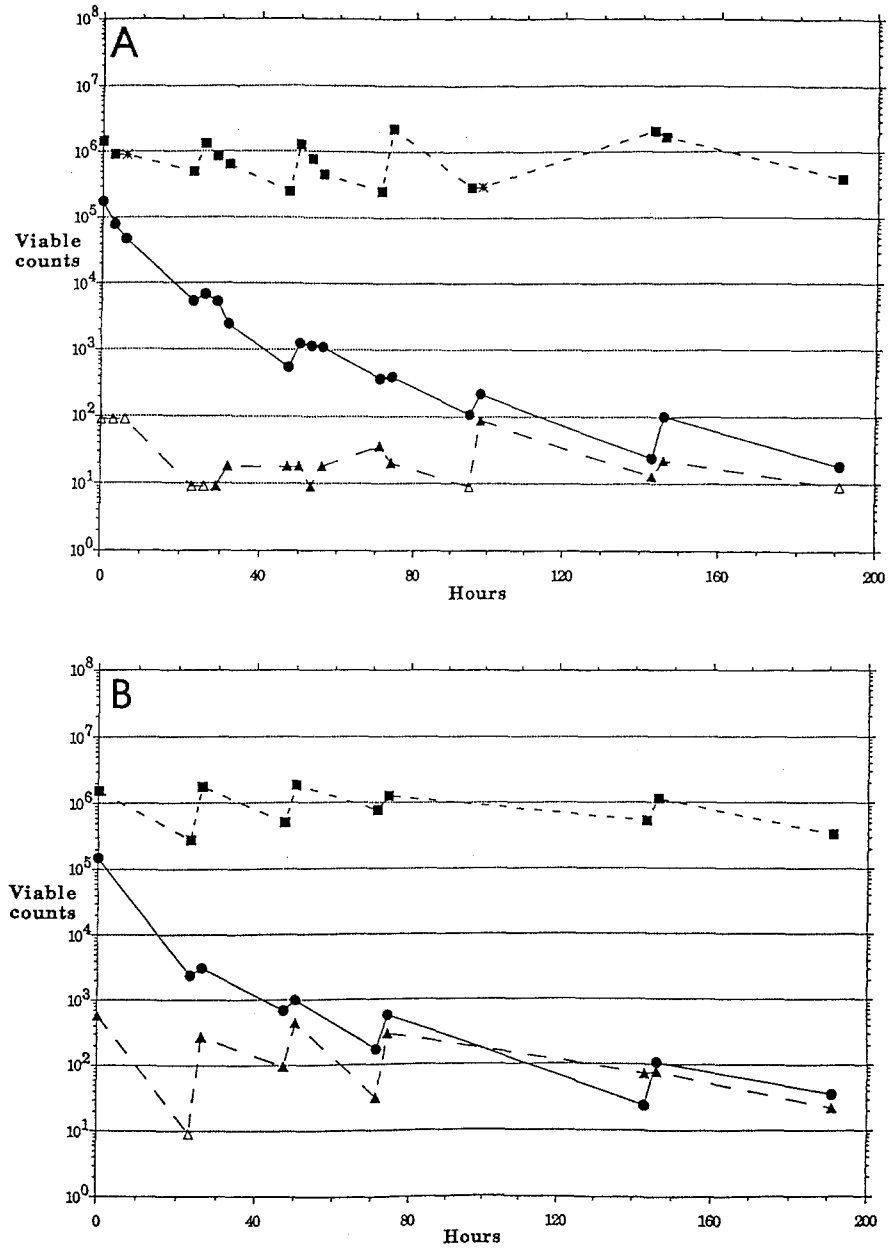

Fig. 5. Microbial counts for experimental trials 1 (panel A) and 2 (panel B) of SCAS units 4, 6, and 8 inoculated with a low concentration of *E. coli* strain LBB270 [pBGH1] and uninoculated SCAS units 1 and 2. The mean total aerobic microbial counts from SCAS units 4, 6, and 8 (– – ■ – –) were determined on TYG plates and includes *E. coli* strain LBB270 [pBGH1] and indigenous populations. The population of *E. coli* LBB270 [pBGH1] in SCAS units 4, 6, and 8 (–●–) and indigenous antibiotic-resistant bacteria in SCAS units 1 and 2 (– – ▲ – –) were determined on EMB antibiotic plates. Some values were greater than the value shown (– – * – –) because there were more than 300 colonies on these plates. Open symbols (– –△– –) indicate that the lowest dilution had no colonies indicating the actual microbial count was lower than these estimates.

centration comparable to the indigenous populations, it was not able to compete and survive. The total viable counts decreased to the point that colonies of *E. coli* LBB270 [pBGH1] could not be distinguished from the indigenous microorganisms resistant to the antibiotics.

These studies were designed to model a 'worst-case' accidental release of a recombinant *E. coli* into a municipal sewage treatment plant. Although highly unlikely, a direct discharge of materials from a fully grown fermenter into an activated sludge plant could add significant levels

of nutrients from the fermentation broth and high levels of recombinant *E. coli*. Although an accidental release to a sanitary sewer would probably undergo significant dilution prior to entering a sewage treatment plant, this study examined both a high inoculum (total dilution about 100-fold) and a low inoculum (total dilution about 10 000-fold). In both cases, the results of this study show that viable counts of recombinant *E. coli* strain LBB270 [pBGH1] in mixed liquor decreased from 10 000 to 100 000-fold in less than 200 h. The *E. coli* discharged in supernatants accounted for less than a 2-fold decrease in the total viable counts, with most of this washout occurring in the first 24-h period. The recombinant *E. coli* failed to survive in any of the inoculated SCAS units.

The results of this study support three general conclusions for the fate of recombinant *E. coli* strain LBB270 [pBGH1] in sewage: (i) The recombinant *E. coli* was not evenly distributed in the mixed liquor, but became preferentially associated with the settlable solids (activated sludge); (ii) The recombinant *E. coli* was unable to maintain its viability in sewage even though it was initially present at concentrations greater than, or comparable to, the indigenous microorganisms; (iii) The recombinant *E. coli* did not become established in sewage. These results are in complete agreement with other published studies [1,2] and demonstrate that derivatives of *E. coli* strain K-12 would not survive in non-sterile sewage.

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