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Death of the *Escherichia coli* K-12 Strain W3110 in Soil and Water

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Whether *Escherichia coli* K-12 strain W3110 can enter the "viable but nonculturable" state was studied with sterile and nonsterile water and soil at various temperatures. In nonsterile river water, the plate counts of added *E. coli* cells dropped to less than 10 CFU/ml in less than 10 days. Acridine orange direct counts, direct viable counts, most-probable-number estimates, and PCR analyses indicated that the added *E. coli* cells were disappearing from the water in parallel with the number of CFU. Similar results were obtained with nonsterile soil, although the decline of the added *E. coli* was slower. In sterile water or soil, the added *E. coli* persisted for much longer, often without any decline in the plate counts even after 50 days. In sterile river water at 37°C and sterile artificial seawater at 20 and 37°C, the plate counts declined by 3 to 5 orders of magnitude, while the acridine orange direct counts remained unchanged. However, direct viable counts and various resuscitation studies all indicated that the nonculturable cells were nonviable. Thus, in either sterile or nonsterile water and soil, the decline in plate counts of *E. coli* K-12 strain W3110 is not due to the cells entering the viable but nonculturable state, but is simply due to their death.

The "viable but nonculturable" (VBNC) hypothesis holds that certain normally culturable bacteria can enter a state in which they are still viable but cannot be cultured by standard microbiological methods (3, 10, 28). The basic observations are as follows. A culturable bacterium is inoculated into a sterile microcosm, most commonly seawater or river water, and incubated for a number of days with regular monitoring. Under certain conditions, it has been observed that as the number of culturable cells declined (enumerated by plate counts), the total number of cells present remained unchanged (enumerated by a direct count method). The central tenet of the VBNC hypothesis is that these nonculturable cells are not dead, but are in the VBNC state.

Papers by proponents of the VBNC hypothesis are often accompanied by statements about the possible consequences of their observations. The general theme of these statements is that bacteria in the VBNC state, particularly in water, pose a potential threat to the public health because they cannot be detected by standard microbiological testing methods (10, 11, 18, 32–34, 37, 39). If this were true, it would follow that developed nations would be awash in unexplained outbreaks of waterborne microbial disease, but this is not the case. In a detailed exploration of this topic, Geldreich (17) has catalogued only 104 outbreaks and 22 deaths from waterborne bacterial disease in the United States over the past 70 years. Furthermore, lengthy case histories repeatedly showed that these outbreaks could be ascribed to causes such as breakdowns of drinking water distribution systems, but never to any inadequacy of standard bacteriological methods.

Issues such as these have made the VBNC hypothesis somewhat controversial (3). For the present study, the question posed was whether the well-characterized, prototypic *Escherichia coli* K-12 strain W3110 enters the VBNC state. The approach was to establish conditions under which this strain was either disappearing or forming large populations of nonculturable cells and then to determine whether these population changes were due to the death of the cells or to development of the VBNC state.

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MATERIALS AND METHODS

Bacterial strains and preparation of inocula. The *E. coli* K-12 strain used was the standard prototrophic wild-type strain W3110 (1). For the studies requiring a plasmid-bearing strain, strain W3110 was transformed with the plasmid pBR322 (2). This particular plasmid was used to permit the inclusion of ampicillin and tetracycline in the direct viable count (DVC) method and to facilitate the PCR measurements employed. Fresh cultures of the strains W3110 and W3110(pBR322) that had been grown for 14 h in Luria-Bertani (LB) medium [plus ampicillin and tetracycline in the case of W3110(pBR322)] were washed with sterile 0.9% saline, adjusted to the desired cell concentration, and added to the water microcosms in 10 ml of inocula or to the soil microcosms in 3 ml of inocula.

Media and chemicals. Levine eosin methylene blue (EMB) agar, tryptone, yeast extract, brain heart infusion medium, and Bacto-agar were obtained from Difco Laboratories (Detroit, Mich.). Cycloheximide, nalidixic acid, acridine orange, 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride (INT), and glycine betaine were obtained from Sigma Chemical Co. (St. Louis, Mo.). LB medium (35) was used to grow the strains; LB agar was LB medium with 15 g of Bacto-agar per liter.

Plating of nonsterile soil suspensions on LB agar gave low total counts, mainly because the colonies were often obscured by mats of fungal growth and because of the tendency of many of the soil bacteria to swarm. Use of soil extract (SEC) agar gave higher total counts, and inclusion of cycloheximide prevented both fungal growth and bacterial swarming. Soil extract was prepared by suspending 1 kg of soil and 0.5 g of calcium carbonate in 1 liter of distilled water. The suspension was autoclaved for 60 min and allowed to settle overnight, and the supernatant was autoclaved for 30 min. SEC agar was prepared by suspending 15 g of Bacto-agar and 0.5 g of dipotassium phosphate in 900 ml of distilled water; after autoclaving for 30 min, 5 ml of sterile (autoclaved) 20% dextrose, 100 ml of soil extract, and 12.5 mg of cycloheximide (dissolved in distilled water and filter sterilized) were added. Both the soil bacteria and *E. coli* grew well on SEC agar, forming robust colonies after incubation for 72 h at 20°C.

Water microcosms. The river water used was obtained from the Missouri River at a site about 19 km upstream of St. Louis, Mo. A detailed chemical and microbiological characterization of this river water has been published elsewhere (5). Artificial seawater was prepared from Instant Ocean (Aquarium Systems, Mentor, Ohio) at a concentration of 3.5% in distilled water. One-liter aliquots of water were placed into 2-liter Erlenmeyer flasks capped with foam plugs and paper covers. For the sterile water studies, the flasks of water were autoclaved for 45 min. After inoculation, the flasks were incubated in the dark at 4, 20, or 37°C. Uninoculated flasks of nonsterile river water were used as controls at all tem-

peratures; at the outset, indigenous bacteria were present at a level of about 3.9×10^3 CFU/ml of river water.

Soil microcosms. The soil used in these studies was obtained from an undisturbed woodland site in rural St. Louis County, Mo. The soil was a silt loam, containing 9% sand, 75% silt, and 16% clay; there was 2.7% organic matter, and the pH of the soil was 6.9. The soil was passed through a 2-mm-pore-diameter sieve, and 27-g aliquots were placed in 125-ml Erlenmeyer flasks capped with rubber stoppers. For the sterile soil studies, the flasks of soil were autoclaved for 45 min on two successive days. After inoculation, the flasks were incubated in the dark at 4, 20, or 37°C. Uninoculated flasks of nonsterile soil were used as controls at all temperatures; at the outset, indigenous bacteria were present at a level of about 2.1×10^7 CFU/g of soil.

Colony and cell counting. Samples for counting were removed directly from the water microcosms or from the soil microcosms after resuspension in 100 ml of sterile 0.9% saline. The samples were diluted in sterile 0.9% saline for subsequent counting. Plate counts were performed by plating 0.1-ml samples in duplicate. EMB plates were incubated at 37°C for 48 h, and LB and SEC plates were incubated at 20°C for 72 h prior to counting. All of the colonies on plates containing less than 300 colonies were added up and divided by the total volume of the original solution to estimate the number of CFU per milliliter. Plate counts on EMB agar were used to enumerate the CFU per milliliter of the *E. coli* strain; plating of 0.1-ml samples of water or soil suspensions yielded a limit of detection of 10 CFU/ml of river water and 100 CFU/g of soil. Plate counts on SEC agar or LB agar yielded the number of CFU per milliliter of all aerobic bacteria (both the added *E. coli* plus the indigenous aerobic bacteria) in the soil or water, respectively. Most-probable-number (MPN) estimates were performed with LB medium with a 10-tube procedure and probability table (22); the tubes were incubated at 37°C for 48 h on a platform shaker at 300 rpm prior to scoring growth. Samples from tubes exhibiting growth were diluted in 0.9% saline, plated on EMB agar, and incubated at 37°C for 48 h to confirm the presence of *E. coli*. Too high a ratio of indigenous bacteria to *E. coli* was found to interfere with the MPN count; spike recovery experiments indicated a limit of detection for the *E. coli* of 10 cells per ml of river water and 1,000 cells per g of soil. Acridine orange direct counts (AODC) were performed by the method of Hobbie et al. (23) with a 0.9% saline diluent and staining with 0.01% acridine orange at room temperature for 15 min; this indicated the total number of cells per ml, regardless of whether they were able to grow into a visible colony. DVC were performed by the method of Kogure et al. (25), with incubation of the samples in 0.025% yeast extract and 0.002% nalidixic acid at room temperature overnight before acridine orange staining. Cells which were elongated to at least twice the length of AODC controls were scored as viable. The diluent was Vogel-Bonner minimal medium (38); when performing the DVC for *E. coli* W3110 (pBR322) in nonsterile river water or soil, ampicillin at 100 μg/ml and tetracycline at 10 μg/ml were included with the samples to prevent the growth or elongation of indigenous bacteria. There were no detectable ampicillin- and tetracycline-resistant indigenous bacteria in either the soil or the water samples. The limit of detection for the AODC and the DVC was 1.7×10^2 cells per ml of river water; since the soil suspensions had to be diluted 1,000-fold before the particle content was low enough to allow cells to be counted, the limit of detection in soil was 1.7×10^5 cells per g of soil. The INT reduction technique of Quinn (30) was also used as a viable count method. A Nikon Optiphot fluorescent microscope with an HBO-100 light source was used for the examination of the preparations at a magnification factor of 1,000.

Isolation of DNA from water and soil. Ten-milliliter aliquots of water were centrifuged at $1,500 \times g$ for 10 min. Plasmid DNA was isolated from the resulting pellet by the alkaline extraction procedure of Birnboim and Doly (4).

A more complex method had to be employed to isolate plasmid DNA from cells in soil. Twenty-seven grams of soil was suspended in 80 ml of lysis solution (25 mM Tris [pH 8.0], 10 mM EDTA, 55 mM glucose containing 250 μg of lysozyme per ml) and placed on ice for 5 min. To 27 ml of this suspension was added 54 ml of alkaline sodium dodecyl sulfate (SDS) solution (0.2 M NaOH, 1% SDS), and the mixture was placed on ice for another 5 min. To this was added 42 ml of 3 M potassium acetate (pH 4.8), and the solution was incubated on ice for another 15 min. Next, 12.5 ml of this mixture was centrifuged at $10,000 \times g$ for 10 min. Isopropanol (7.5 ml) was added to the supernatant, and this was placed in a dry ice and ethanol bath for 10 min. After thawing, the solution was centrifuged at $10,000 \times g$ for 10 min. The resulting pellet was washed with cold 70% ethanol and dried in a vacuum desiccator for 15 min. The pellet was resuspended in 100 μl of TE buffer (10 mM Tris [pH 8.0], 1 mM EDTA) and passed over a Qiagen, Inc. (Chatsworth, Calif.), P-20 column as described in the kit instructions provided by the manufacturer. The eluate was subjected to a Promega Corporation (Madison, Wis.) Wizard DNA Clean-up procedure as described in the kit instructions provided by the manufacturer, and the resulting DNA solution was ready for PCR analysis.

PCR assay method. The target DNA used in these studies was the plasmid pBR322 (2). The primers PBR1 (5′-GCGACCGAGTTGCTCTTGCCCGGCG-3′) and PBR2 (5′-GCAAGGAGATGGCGCCCAACAGTCC-3′), obtained from Midland Certified Reagent Co. (Midland, Tex.), were used in a PCR assay to amplify a 1,042-bp fragment of pBR322. The PCR was carried out with a DNA thermal cycler and reagent kit purchased from Perkin-Elmer Cetus (Norwalk, Conn.) at a magnesium chloride concentration of 3 mM. The samples were subjected to a total of 30 PCR cycles, with 10 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min, followed by 20 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 2 min. The resulting PCR products were analyzed by electrophoresis on a 7% polyacrylamide gel followed by staining with ethidium bromide. To estimate the relative concentration of the pBR322 template, each sample was diluted in a series of 10-fold steps, and each dilution was assayed three times by the PCR. These dilutions were carried out to the point where there was no longer plasmid DNA template present to be amplified by PCR. The results were treated as an MPN test with a three-tube probability table (9). Spiking soil samples with various numbers of *E. coli* W3110(pBR322) cells yielded a standard curve with a limit of detection of 300 cells per g of soil.

RESULTS

Decline of *E. coli* in nonsterile soil and water. Despite being introduced into the nonsterile soil and water at very high levels, the added *E. coli* strains were completely lost over the course of these studies. At all three temperatures tested (4, 20, and 37°C), the added *E. coli* rapidly declined to levels undetectable by plate counts on EMB agar or by MPN estimates or DVC (Fig. 1). A similar result was obtained with strain W3110 (pBR322) in the nonsterile soil microcosms, although the decline was not as rapid (Fig. 2).

In all cases, the total plate counts per milliliter of river water on LB agar or per gram of soil on SEC agar were the same as the *E. coli* plate counts on EMB agar as long as the populations of *E. coli* were higher than those of the indigenous bacteria (Fig. 1 and 2). The correlation between the plate counts on selective and nonselective media was lost once the populations of inoculated *E. coli* dropped below the levels of indigenous bacteria. At this point, the indigenous bacteria became the major fraction of the bacterial population and remained so for the duration of the experiments. The AODC also paralleled the plate counts on LB or SEC agar, indicating that as the *E. coli* plate counts declined, the total number of *E. coli* cells was also declining. The MPN and DVC of *E. coli* cells declined in parallel with the plate counts, indicating that once the populations of *E. coli* dropped below the indigenous bacterial populations, the AODC counts were tracking only the indigenous bacteria.

An additional indication of the loss of *E. coli* cells from the river water microcosms was provided by turbidity measurements. Spectrophotometric monitoring showed that the turbidity of the river water declined in parallel with the decline in the *E. coli* populations and levelled off at the preinoculation turbidity once the populations of inoculated *E. coli* cells dropped below the levels of indigenous bacteria.

The PCR was used to monitor the presence of plasmid-containing *E. coli* cells in the nonsterile soil and water. Plasmid DNA samples were isolated at each time point from one 20°C soil microcosm and one 20°C river water microcosm, and the degree to which they could be diluted and still give a positive PCR signal was determined. Spike recovery experiments demonstrated that this method yielded good approximations of the number of *E. coli* cells remaining. In both cases, the relative concentration of the pBR322 template decreased in parallel with the other *E. coli* cell measurements (Fig. 3). Plasmid DNA samples were also isolated from all of the other microcosms at the end of the studies, and in each case, the concentration of pBR322 had dropped below the detection limit.

One possible explanation for the decrease in the relative concentration of the pBR322 template is that the *E. coli* cells were not maintaining the plasmid. In addition, a possible explanation for the decline in *E. coli* EMB plate counts was that after several days in soil or water, the *E. coli* cells were impaired in their ability to grow on the EMB medium. These possibilities were eliminated by replica-plating experiments. As described above, samples of the soil and water microcosms

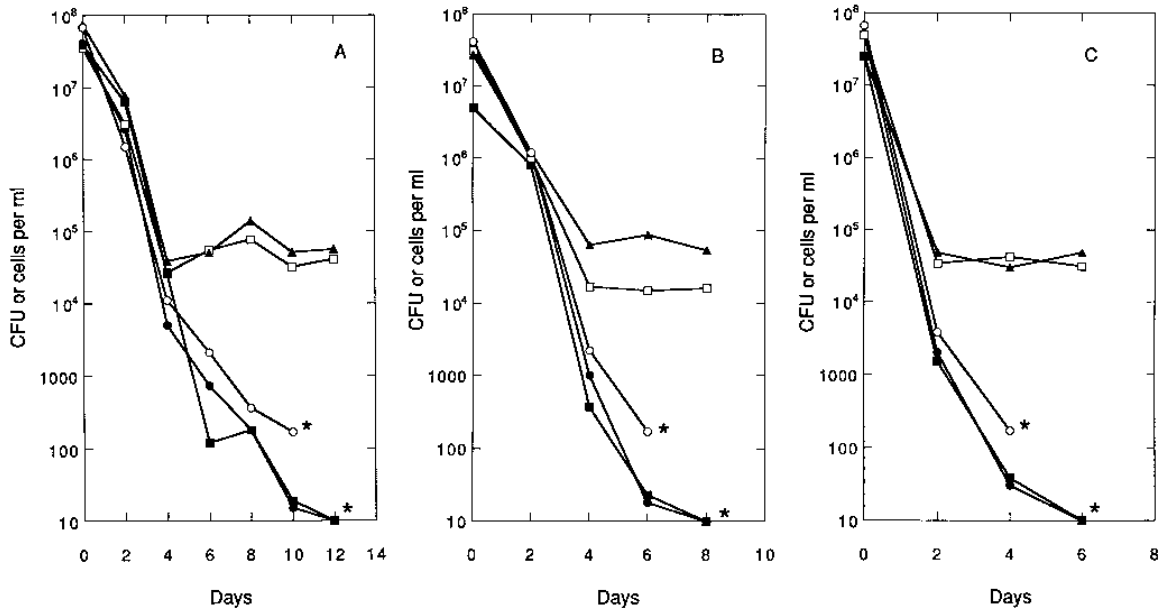

FIG. 1. Decline of *E. coli* cells in nonsterile river water at 4°C (A), 20°C (B), and 37°C (C). Data were obtained from studies with strain W3110(pBR322). Counts of *E. coli* CFU per milliliter of river water by plate counts on EMB agar (●), *E. coli* viable cells per milliliter by MPN estimate (■) and DVC (○), total CFU per milliliter by plate counts on LB agar (□), and total cells per milliliter by AODC (▲) are shown. Each point is the mean of values from duplicate microcosms. Standard errors were approximately 14, 18, 10, and 9% of the means for plate counts, MPN estimates, DVC, and AODC, respectively. The last points plotted for the *E. coli* plate counts, MPN, and DVC are marked with an asterisk to indicate that they are at the limit of detection for those methods on the days when the respective counts fell below that limit.

were plated on EMB agar and on LB or SEC agar in parallel. At each time point, single representative LB or SEC plates were replica plated to EMB media without antibiotics and also to EMB media containing ampicillin at 100 μg/ml and tetracycline at 10 μg/ml; in all cases, the LB and SEC plates were found to have numbers of *E. coli* colonies similar to those of the corresponding original EMB plates, and of 200 to 300 *E. coli* colonies tested at each time point, all had retained the plasmid. Also at each time point, single original EMB plates were replica plated to EMB with ampicillin and tetracycline,

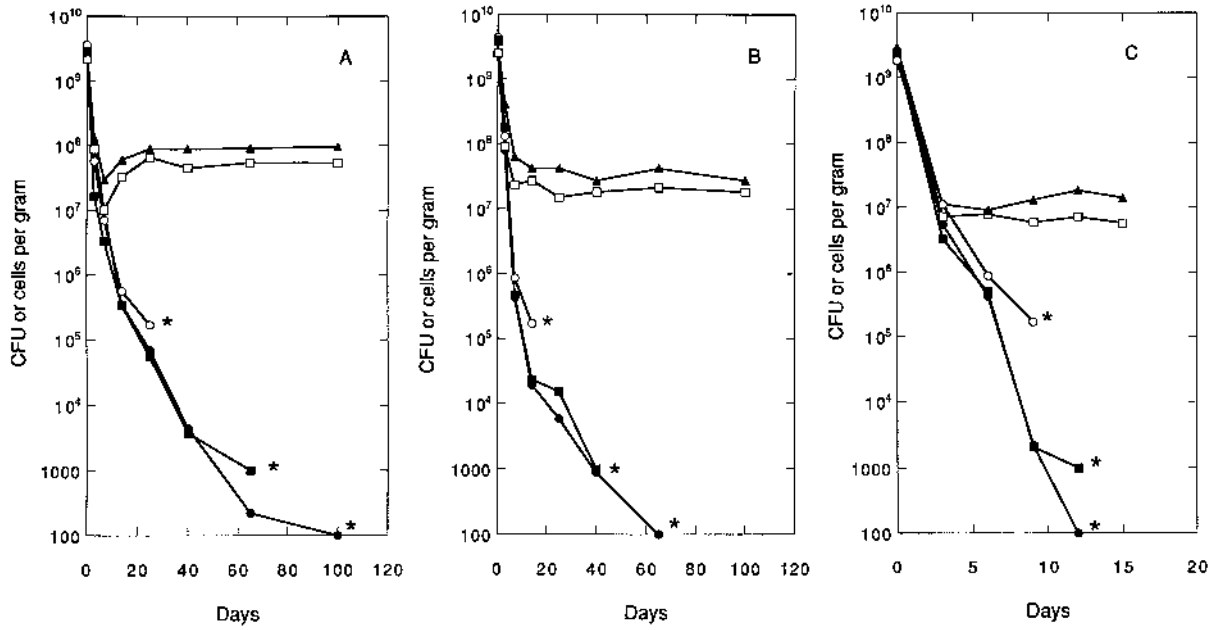

FIG. 2. Decline of *E. coli* cells in nonsterile soil at 4°C (A), 20°C (B), and 37°C (C). Data obtained from studies with strain W3110(pBR322). Counts of *E. coli* CFU per gram of soil by plate counts on EMB agar (●), *E. coli* viable cells per gram by MPN estimate (■) and DVC (○), total CFU per gram by plate counts on SEC agar (□), and total cells per gram by AODC (▲) are shown. Each point is the mean of values from duplicate microcosms. Standard errors were as given in the legend to Fig. 1. The last points plotted for the *E. coli* plate counts, MPN, and DVC are marked with an asterisk to indicate that they are at the limit of detection for those methods on the days when the respective counts fell below that limit.

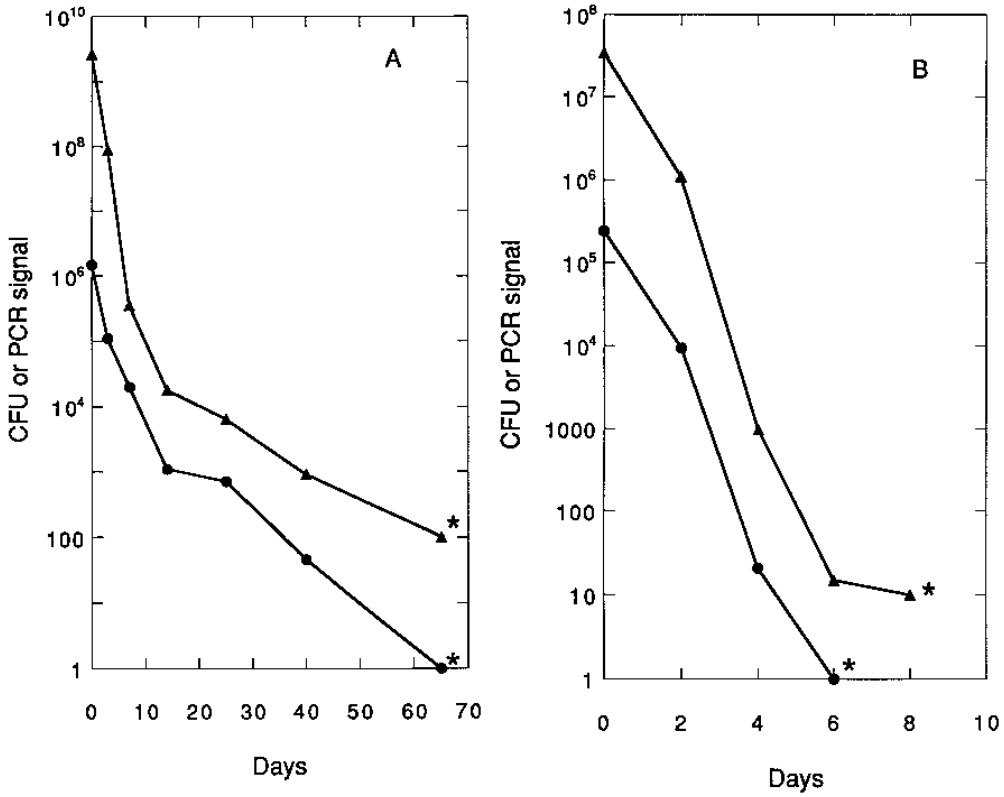

FIG. 3. Decline of PCR signal strength and *E. coli* plate counts in nonsterile soil at 20°C (A) and nonsterile river water at 20°C (B). Data were obtained from studies with strain W3110(pBR322). Counts of *E. coli* CFU per gram of soil or per milliliter of river water by plate counts on EMB agar (▲) are shown. PCR signal strength, expressed as the greatest dilution of isolated plasmid DNA which yielded an amplified fragment, was calculated as a three-tube MPN estimate (●). For example, a value of 100 indicates that the plasmid DNA sample could be diluted up to 100-fold and still yield an amplified fragment. Each point is the mean of values from duplicate microcosms. Standard errors were approximately 14 and 24% of the means for plate counts and PCR signal strengths, respectively. The last points plotted are marked with an asterisk to indicate that they are at the limit of detection for those methods on the days when the respective values fell below that limit.

and all of these colonies (200 to 300 per plate) were observed to have retained the plasmid.

It became difficult to track the plasmid-free strain W3110 once the populations of this strain had fallen below the levels of indigenous microorganisms in the nonsterile soil or river water. The PCR assay could not be used, since it was designed to use the plasmid DNA as template; attempts to develop a PCR assay with chromosomal DNA as a template were thwarted by interference from the chromosomal DNA of the indigenous microorganisms, resulting in a very high limit of detection. The DVC could not be used, since ampicillin and tetracycline (which the plasmid imparts resistance to) could not be employed to inhibit the growth or elongation of the indigenous microorganisms, making it impossible to distinguish between the added *E. coli* cells and the indigenous microorganisms. However, the other monitoring methods indicated that strain W3110 declined in a manner essentially identical to that of strain W3110(pBR322). The total plate counts were the same as the *E. coli* plate counts as long as the populations of *E. coli* were higher than those of the indigenous bacteria. The AODC also paralleled these plate counts, indicating that as the *E. coli* plate counts declined, the total number of *E. coli* cells was also declining. After the populations of strain W3110 had fallen below the levels of indigenous microorganisms, they could still be monitored by *E. coli* plate counts and MPN estimates. These counts exhibited a continued decline similar to that of strain W3110(pBR322) illustrated in Fig. 1 and 2.

Viability studies with *E. coli* in sterile soil, sterile river water, and sterile artificial seawater. The complications from the presence of indigenous microorganisms, discussed in the paragraph above, were not encountered in the sterile soil or water microcosms. Both of the *E. coli* K-12 strains W3110 and W3110(pBR322) were easy to monitor in the sterile microcosms. Under most conditions in sterile soil or water, it was observed that the plate counts on LB agar of the two *E. coli* strains remained at the initial levels for extended periods of time. In sterile soil at 4, 20, and 37°C, the *E. coli* strains did not exhibit any decline in plate counts even after 100 days. In sterile artificial seawater at 4°C or sterile river water at 4 or 20°C, the *E. coli* plate counts remained constant for over 50 days. Replica-plating tests at the end of the studies with strain W3110(pBR322) indicated that the plasmid had been retained.

Three conditions in sterile water were identified in which the *E. coli* viable and culturable cell counts did decline: sterile river water at 37°C and sterile artificial seawater at 20 and 37°C. Four different methods were used to monitor the *E. coli* cells in these three microcosms: plate counts on LB agar, MPN estimates, AODC, and DVC. As measured by AODC, the number of *E. coli* cells remained constant at the initial level in these waters, but the plate counts, MPN estimates, and DVC all gradually declined in parallel over the 56 days of the studies (Fig.4). The strains W3110 and W3110(pBR322) exhibited nearly identical death curves under these conditions; replica-plating studies indicated that the surviving cells of W3110(pBR322) had all retained the plasmid.

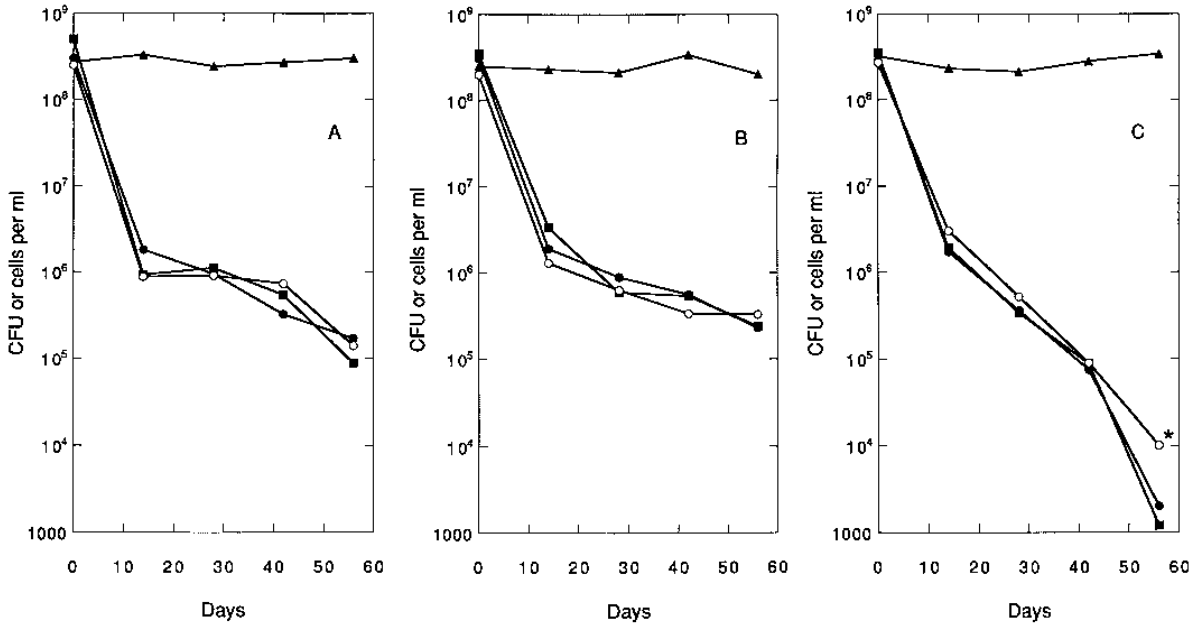

FIG. 4. Decline of *E. coli* cells in sterile river water at 37°C (A) and sterile artificial seawater at 20°C (B) and 37°C (C). Data were obtained from studies with strain W3110(pBR322). Counts of *E. coli* CFU per milliliter of water by plate counts on LB agar (●), *E. coli* viable cells per milliliter by MPN estimate (■) and DVC (○), and total cells per milliliter by AODC (▲) are shown. Each point is the mean of values from duplicate microcosms. Standard errors were as given in the legend to Fig. 1. The limit of detection for the DVC in these samples was about 10^4 cells per ml, since the large number of dead *E. coli* cells obscured the field when less dilute samples were examined. The last point plotted for the DVC in the sterile artificial seawater at 37°C is marked with an asterisk to indicate that it is at this limit of detection.

The INT reduction technique for counting viable cells was also employed in these studies as another type of viable cell count. At the initial time points (days 0 and 14), the INT counts were approximately the same as the plate counts, the MPN estimates, and the DVC. However, at the later time points, the INT counts were 2 to 3 orders of magnitude lower than the counts obtained by the other three methods, so the INT reduction counting technique was not considered accurate.

Resuscitation studies. The nonculturable fraction of cells from the 37°C sterile river water and 20 and 37°C sterile artificial seawater microcosms could not be resuscitated by any of a variety of techniques. The MPN method was actually one type of resuscitation technique, because the cells were diluted and incubated in LB medium at 37°C for 48 h. However, the MPN results consistently confirmed the losses shown in the plate count and DVC results (Fig. 4).

The viable and culturable cell counts had dropped by more than 100-fold after 14 days in these three microcosms (Fig. 4). The MPN tubes without growth were incubated for an additional 48 h but still exhibited no growth. The MPN tubes that did exhibit growth had always grown up to full density, ranging from 2×10^9 to 5×10^9 CFU/ml. Representative MPN tubes at the lowest dilutions without growth, which would have been inoculated with no culturable cells but about 100 nonculturable cells, were also examined by AODC and DVC and found to contain no detectable cells (the limit of detection for the AODC and DVC under these conditions was 1.7×10^2 cells per ml). This indicated that no resuscitation and growth of the nonculturable cells had occurred. After 56 days in these three microcosms, the viable and culturable cell counts had declined by more than 1,000-fold (Fig. 4). The MPN resuscitation tests again confirmed the plate counts and DVC results. Tubes at the lowest dilutions without growth in these cases would have been inoculated with no culturable cells but about 1,000 nonculturable cells (about 100,000 nonculturable cells in the case of sterile artificial seawater at 37°C); after an additional 48-h incubation, these numbers were confirmed by AODC and DVC, with none of the cells in the DVC samples giving any indication of viability (they were not elongated compared with the AODC samples). Again, no resuscitation and growth of the nonculturable cells had occurred.

To test whether the LB medium was too lean for resuscitation to occur, a richer brain heart infusion medium was also used on days 14 and 56 of these studies; the MPN results were essentially the same in this medium as in LB medium. Alternatively, the LB medium could have been too rich for resuscitation. To test this possibility, 4% LB medium and 20% LB medium were also employed on days 14 and 56, with results essentially the same as those obtained with full-strength LB medium.

Also on days 14 and 56, tubes of LB medium that had been inoculated with small numbers (about 10) of culturable cells but much larger numbers of nonculturable cells were monitored for the first few hours of incubation by plating of 0.1-ml samples on LB agar. There was a gradual increase in the plate counts in these tubes, indicating growth and division of the small numbers of culturable cells rather than a rapid resuscitation of the much larger numbers of nonculturable cells.

Since the *E. coli* populations remained unchanged in the sterile water microcosms at 4°C, the effects of temperature shifts on the warmer microcosms were investigated. On days 14 and 42, samples of the 37°C sterile artificial seawater and 37°C sterile river water microcosms were placed at 4 and 20°C, and samples of the 20°C sterile artificial seawater microcosm were placed at 4°C. After 24 and 72 h at these lower temperatures, there was no change in the number of viable cells as measured by AODC, plate counts, and MPN estimates.

Osmotic stress was considered as an alternative explanation of the apparent death of the *E. coli* cells in the 20 and 37°C

artificial seawater. On days 14, 28, and 42, samples from both of these microcosms were diluted in either distilled water, 0.9% saline, 3.5% saline, or artificial seawater and plated on standard LB agar (which contained 10 g of sodium chloride per liter), LB agar with the sodium chloride content increased to 35 g/liter, and LB agar with the sodium chloride replaced by 35 g of artificial seawater salts per liter. A control culture of the *E. coli* K-12 strain W3110 grown overnight in LB medium was treated in the same manner. In all cases, the plate counts were unaffected by either the diluent or the salt content of the LB agar. Holding the cells for 6 h in the various diluents before plating also did not affect the plate counts. It has been reported that the compound glycine betaine can restore plate counts in osmotically stressed *E. coli* (34); incubation of samples on days 28 and 56 from the 20 and 37°C sterile artificial seawater microcosms in 2, 6, or 20 mM glycine betaine for 6 and 24 h did not affect the plate counts. These results indicated that the *E. coli* cells in these artificial seawater microcosms were not under osmotic stress.

DISCUSSION

Under all of the conditions employed in these studies, the *E. coli* strains did not appear to enter the VBNC state. The results reported here were obtained from 60 separate viability studies, involving two *E. coli* K-12 strains [W3110 and W3110 (pBR322)] in five types of microcosms (nonsterile soil and river water and sterile soil, river water, and artificial seawater) at three temperatures (4, 20, and 37°C), with each study performed in duplicate.

In the nonsterile soil and river water microcosms, the *E. coli* cells dropped below the limit of detection of every technique used to monitor them. For the initial stages of each of these studies, the AODC provided a direct indication that the total cell counts of the added *E. coli* cells were declining in parallel with the culturable cell counts (Fig. 1 and 2). Once the *E. coli* cell counts fell below the level of indigenous bacteria, the AODC levelled off (since from that point on, the cells of the indigenous bacteria were the only ones being counted by the AODC), but all of the other *E. coli*-specific monitoring methods indicated that the *E. coli* cells continued to disappear. The most direct indications of this continued decline were the PCR measurements showing the disappearance of the plasmid pBR322 in parallel with the drop in viable cell counts (Fig. 3).

These observations indicated that *E. coli* cells added to nonsterile soil or river water disappeared completely, suggesting that they were unable to compete with and perhaps were being consumed by the indigenous inhabitants of these environmental samples. These findings are consistent with earlier reports that the decline of *E. coli* populations in water was due to predation by protozoa (19, 21, 36) and exposure to heat-labile toxins (24). In the microcosms with added *E. coli* cells, the indigenous populations of bacteria increased slightly over the course of these studies (Fig. 1 and 2), perhaps as a result of nutrients provided by death of the *E. coli* cells; the indigenous bacterial populations did not change in uninoculated soil or river water.

In the sterile water and soil microcosms studied here, the plate counts of the *E. coli* strains were found to remain constant at the initial level in sterile soil at 4, 20, and 37°C; in sterile river water at 4°C and 20°C; and in sterile artificial seawater at 4°C. In sterile river water at 37°C and sterile artificial seawater at 20 and 37°C, the AODC remained constant at the initial level, while all other indications were that the number of viable and culturable cells was dropping (Fig. 4). These results indicated that the nonculturable fractions of *E. coli* cells were nonviable and that these *E. coli* K-12 strains did not enter the VBNC state. It has been suggested that within a short time (about 4 days) after apparent die-off, cells in the VBNC state can be resuscitated by incubation in a rich medium (31, 33). However, repeated resuscitation attempts (dilution and incubation in LB medium for the MPN estimates) over the entire course of these studies indicated that the nonculturable cells were not able to be resuscitated. There was also no indication that the cells could be resuscitated by a temperature downshift or that the cells in the sterile artificial seawater were under any osmotic stress which could account for their decline.

It could not be ruled out from the data obtained in these studies that the *E. coli* cells could pass through a transient VBNC state before dying. However, the close agreement between the culturable cell counts (plate counts and MPN estimates) and the viable cell counts (Fig. 1, 2, and 4) indicates that the level of any such transient cells was undetectable. Since this was true over the entire course of the studies presented here, such transient cells (if they even existed at all) did not persist and accumulate to detectable levels, but died off in a very short period of time.

It is reasonably clear that both *E. coli* strains W3110 and W3110(pBR322) did not enter the VBNC state in sterile soil or water. The studies with the nonsterile river water or soil were more complicated. The indigenous bacteria reduced the sensitivity of the colony and cell counting techniques, and the DVC and PCR techniques could only be used with the plasmid-containing strain. However, the results suggest not only that both *E. coli* strains did not enter the VBNC state, but also that the *E. coli* cells were disappearing from the nonsterile soil and water; this was particularly clear from the declining AODC counts for both strains as long as the populations of *E. coli* cells were greater than those of the indigenous microorganisms (Fig. 1 and 2). Once the *E. coli* populations dropped below the level of indigenous microorganisms, culturable cell counts continued to decline for both strains; for the plasmid-containing strain, the DVC and PCR measurements paralleled these declines (Fig. 1 to 3), indicating that this strain was not entering the VBNC state. However, the DVC had a higher limit of detection and could not be used for the entire duration of the studies (Fig. 1 and 2). These monitoring difficulties raise the question of whether the plasmid free strain could have entered the VBNC state some time after the *E. coli* populations had dropped below the level of indigenous microorganisms and whether the plasmid-containing strain could have entered the VBNC state some time after the DVC limit of detection was passed. Related questions are whether the plasmid-containing strain survived better than the parent strain in these microcosms and whether plasmid-free segregants of W3110(pBR322) had arisen and immediately entered the VBNC state (since no plasmid-free segregants were observed in the replica-plating tests). Such possibilities require conjecturing that the plasmid pBR322 impairs the ability of strain W3110 to enter the VBNC state while at the same time improving the survivability of the strain, that this strain only enters the VBNC state in nonsterile (but not in sterile) soil or water, and that entry into the VBNC state is delayed until it is undetectable. These possibilities also require discounting the trend of continued decline of both strains seen in all of these studies. While the possibility of the existence of undetectable VBNC cells cannot be ruled out, the most parsimonious explanation of the results presented here is that these strains did not enter the VBNC state.

There have been conflicting reports on the survival of *E. coli* in water from other studies. Considering first the studies performed with *E. coli* K-12 strains (6, 8, 13, 15, 16), the trend that

emerges is that temperatures of at least 37°C in sterile freshwater and at least 15°C in sterile seawater are required for *E. coli* K-12 strains to exhibit a decline in plate counts. However, with non-K-12 strains of *E. coli*, there is no clear pattern (7, 12, 14, 18, 20, 26, 27, 29, 36, 37). Most of these studies do report that at 5°C, declines in plate counts occur in sterile freshwater or sterile seawater, but not at warmer temperatures; this is in complete contrast to the studies involving *E. coli* K-12 strains.

The conclusion from the studies presented here is that *E. coli* K-12 strain W3110 does not enter the VBNC state. The present studies were broad in their scope and in the array of techniques employed and yielded consistent and uniform results. The results further suggest that the *E. coli* cells were disappearing from the nonsterile microcosms studied (perhaps being consumed by indigenous microorganisms), raising the question of whether the VBNC state would be irrelevant in natural environments. The strains were stable in sterile soil even at 37°C; others have observed that soil and sediment protect and stabilize *E. coli* cells (12, 14). Why the *E. coli* strains were dying in the warmer sterile water microcosms is not known, but may be due to a temperature-dependent starvation-induced phenomenon without relevance to natural environments.

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