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Retention of Enteropathogenicity by Viable but Nonculturable *Escherichia coli* Exposed to Seawater and Sunlight

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The effect of natural sunlight on culturability and persistence of pathogenicity of *Escherichia coli* was examined in the field, i.e., in the Morlaix Estuary, France, using an enterotoxigenic strain of *Escherichia coli* H10407. Results showed that *E. coli* responds to the estuarine diurnal solar cycle by entering the viable but nonculturable state upon exposure to sunlight. That is, direct counts of viable cells remained stable without significant change, but *E. coli* cells remained fully culturable only when exposed to seawater in control chambers in the dark, i.e., without solar irradiation. The effect of sunlight on the pathogenicity of *E. coli* H10407 was studied, using both the rabbit intestinal loop assay and ganglioside-enzyme-linked immunosorbent assay (GM1-ELISA), a sensitive procedure for testing for production of enterotoxin. Results of the GM1-ELISA demonstrated that strains of *E. coli*, after exposure to sunlight and entering the viable but nonculturable state, as well as culturable *E. coli*, retained pathogenicity, i.e., produced enterotoxin. The GM1-ELISA is concluded to be more sensitive than the rabbit intestinal loop assay for analysis of enterotoxin in natural water samples.

Recently, evidence for retention of viability of human pathogens entering the aquatic environment, despite inability to be cultured, has accumulated (18, 19). The assumption has been that human pathogens and indicator organisms die-off (4); however, it has been shown that cells undergoing die-off remain intact and can retain plasmids associated with virulence (3, 8), enteropathogenicity (5, 10), and other functions (22) as well. Because of the increasing use of estuaries, coastal waters, and the open ocean as a repository for domestic wastes, a question with respect to the safety of such practices can be raised, especially the ability of pathogens entering seawater to retain viability (7). Direct detection of *Salmonella* spp. in waste water and estuarine samples from which *Salmonella* spp. could not be isolated in culture (13) indicates a closer examination of the persistence of pathogens in the marine environment is warranted and, further, that methods for their detection, other than plate counts, should be employed. Long-term survival of indicator organisms, e.g., *Clostridium perfringens* (2, 15), at a New York City offshore site where sewage sludge is discharged from barges towed to a deep ocean dump site has been demonstrated (2). Thus, the potential virulence of human pathogenic bacteria and viruses persisting in the ocean becomes relevant (15, 24).

The objective of the study reported here was to examine the effects of sunlight, under normal diurnal cycles, on the pathogenicity of *Escherichia coli*, in view of the currently accepted notion that exposure to sunlight and elevated salinity of seawater offers a purification procedure, as shown by Barcina et al. (1) and Davies et al. (6). The persistence of *E. coli* pathogenicity has previously been reported to occur after exposure to seawater (5, 10), as well as fresh water (23), but it is important to document the effect of exposure to both seawater and sunlight.

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MATERIALS AND METHODS

Bacterial strains and media. *E. coli* H10407 (LT, ST, CFA/I) (7) and HB101 were provided by B. Joly, Faculté de Pharmacie, Clermont-Ferrand, France, and were used in earlier studies to examine survivability in seawater (7). Cultures were prepared in 100 ml of Trypticase soy broth (BBL, Cockeysville, Md.) by incubation from stock broth cultures and incubated for 24 h at 37°C. After harvesting by centrifugation, the cells were washed three times in autoclaved salt solution, i.e., physiological saline (NaCl, 9 g/liter). Culturable counts were obtained by plating appropriate dilutions on Drigalski agar (Oxoid) and incubating the plates for 24 h at 37°C. Estimates of viable cells were also made by direct counts of seawater samples (see below) to which were added 0.025% (wt/vol) yeast extract (Merck, Darmstadt, Germany) and 0.012% nalidixic acid (Sigma Chemical Co., St. Louis, Mo.), by the method of Kogure et al. (14).

For ganglioside-enzyme-linked immunosorbent assay (GM1-ELISA) analyses, Mundell's Casamino Acids medium (16) was employed.

Antiserum. Antiserum to *Vibrio cholerae* toxin was produced, as described by Svennerholm et al. (25). Adult rabbits (Fauve de Bourgogne, France) received three subcutaneous immunizations of 25 mg of purified cholera toxin at 2-week intervals, with the initial two immunizations employing Freund complete adjuvant as diluent. Intracardiac puncture was performed 1 week after the last injection.

The serum was freeze-dried in 0.5-ml portions and stored at −20°C, as described by Svennerholm et al. (25), until needed, when it was dissolved in saline prior to use.

Microcosm experiments. Experiments were carried out using six 1-liter flasks containing ca. 800-ml samples of water collected at high tide from the surface water of the Morlaix Estuary (34.8‰ salinity). The seawater samples were sterilized by passage twice through 0.22-mm-pore-size, 47-mm-diameter membrane filters (Millipore Corporation, Bedford, Mass.). *E. coli* H10407 was inoculated into each flask to a concentration of ca. 10^6 cells per ml. Three of the flasks were exposed to sunlight in a plexiglass water bath at 16 ± 2°C, and three other flasks were also placed in the water bath, but in the dark, i.e., covered with sterile aluminum foil. After incubation for 3, 6, 9, 22, 26, 32, and 46 h, ca. 10-ml volumes were removed from each flask for determination of bacterial counts, using the methods described above.

Field studies were also carried out in the Morlaix Estuary during June 1988, using membrane chambers as described by Fliermans and Gorden (9). In these experiments, quartz chambers, allowing UV light to penetrate, and plexiglass chambers, open to visible light but not to UV, were used to study the effect of the sunlight radiation (UV and visible light). The 600-ml membrane chambers were washed with 95% (vol/vol) ethanol and air dried, after which vacuum was applied. The chambers were filled with seawater, sterilized in situ by filtration using 0.22-μm filters. Immediately prior to submersion, the chambers were inoculated with 1 ml of a suspension of *E. coli* H10407 to a final concentration of 2.5×10^6 cells per ml. The two quartz chambers and the two plexiglass chambers were submerged 0.3 m below the surface, supported by flotation buoys. Bottom experiments were conducted using four plexiglass chambers suspended 0.5 m from the bottom in water of ca. 9 ± 3 m depth. The entire assembly was lowered into

the Morlaix Estuary at the test site. At the Morlaix Estuary site where the experiments were done, the average water temperature was 15°C during the night and 16°C during the day and the salinity was 35 ± 0.5‰.

For bacterial enumerations, samples were collected immediately after inoculation, at 16, 30, 40, 53, and 57 h from the bottom chambers, and at 3, 6, 9, 12, and 34 h from the surface chambers. The samples (10 ml) were collected using a sterile 25-ml syringe.

Upon termination of each experiment carried out in the field, ca. 200 ml of sample was removed from the chambers for the rabbit intestinal loop assay, as follows. All samples used in the experiments were transported to the laboratory on ice, i.e., at ca. 4°C, with total time elapsing from collection to analysis being 6 h or less. In the laboratory, rabbit loops were inoculated with 1.0 ml of sample, without concentration, as follows. Sample 1 consisted of sterile seawater in an uninoculated surface chamber, sample 2 was seawater from the bottom chamber, and sample 3 was seawater from a surface chamber. Sample 4 was a 1/1,000 saline dilution of broth used in the experiments, serving as control.

Light irradiance. Light irradiance was measured using a submersible quantum sensor (LI-COR), permitting detection of radiation in the 400 to 700 range. Light irradiance was measured in the field, just below the surface, and just above the bottom at the time of sample collection. In the laboratory studies, light irradiance was monitored in the uninoculated flask serving as control.

Bacterial enumeration. Culturable bacteria were enumerated as described above. In addition, acridine orange direct counts (AODC) were performed (11), as well as direct counts of viable cells (DVC) (14). For DVC, cells were incubated in the presence of yeast extract and nalidixic acid and subsequently stained, using acridine orange, for counting by epifluorescence microscopy, as described above. Only those cells demonstrating elongation and a reddish-orange fluorescence were counted. DVC and AODC values were calculated for each sample, each value representing the mean of 250 cells or 50 fields.

The counts from spread plates, prepared in duplicate, were arithmetically averaged. When several flasks or chambers were used in an experiment, mean and standard error were calculated for the flask or chamber replicates.

Rabbit intestinal loop assay. Ligated intestinal loops were prepared and inoculated, using male Fauve de Bourgogne rabbits weighing an average of 2.0 kg. Two rabbits, i.e., experiments in duplicate, were maintained without food for 48 h prior to surgery. Employing aseptic techniques and general anesthesia (ethyl carbamate) for each rabbit, a midline incision of ca. 15 cm was made along the abdomen through muscle and peritoneum. The ileum was externalized and four test loops, beginning just above the stomach and 10 cm in length, were tied and separated by three control loops 5 cm in length. The inoculum, 1.0-ml volume, was injected into test loops adjacent to the tie nearest to the appendix, with the second tie isolating the site of inoculation.

The abdominal cavity was filled with sterile NaCl solution (0.9% [wt/vol]) to prevent desiccation during surgery. Temperature (39°C) and anesthesia were carefully controlled during incubation. Rabbits were sacrificed after 7 h, and the abdomen was reopened, externalized, and examined for fluid accumulation (weight/length). A sample of fluid was collected from each loop and tested directly, using GM1-ELISA and again after the addition of 1 to 7 ml of Casamino Acids medium (16), following incubation for 24 h at 37°C, with shaking.

GM1-ELISA procedures. Microtiter ELISA plates (Sterilin, Teddington, England) were coated with 0.2 ml per well of 2 μg of GM1 per ml in phosphate-buffered saline (PBS) and incubated at room temperature (ca. 20°C) for 18 h. The coated plates were stored at 4°C for up to 1 month before use. After washing three times in PBS, the remaining binding sites were blocked by incubating the plates with 1% (wt/vol) bovine serum albumin (BSA) in PBS at 37°C for 1 h (0.2 ml per well). The plates were washed three times with PBS containing 0.2% (vol/vol) Tween 20, after which the samples were added (0.2 ml per well) and incubated for 90 min at 37°C. After a second washing procedure using PBS-Tween 20, 0.2 ml of rabbit serum (anti-cholera toxin diluted 1:1,000 in a solution of PBS–Tween–0.5% BSA) was incubated in wells under the same conditions. After a final washing with PBS-Tween, 0.1 ml of *p*-nitrophenyl phosphate (pNPP; Sigma Chemical Co.) diluted 1 mg/ml in substrate buffer was added to all wells and incubated for 45 min at room temperature. Optical densities were measured using a ELISA plate reader.

RESULTS AND DISCUSSION

The effect of solar irradiation on the viability, i.e., platability, of *E. coli*, as measured in microcosms and in membrane chambers in the field is shown in Fig. 1 and 2. The measurements graphically displayed in Fig. 1A and 2A represent light intensity, expressed as $\mu Em^{-2}S^{-1}$, measured in the laboratory or in the field, the latter just below the surface of the water, measured by a sensor calibrated prior to use by the manufacturer (LI-COR).

E. coli cells exposed to measured quantities of sunlight showed stable DVC and AODC counts, but plate counts (culturable cells) decreased from 2×10^6 cells per ml to no detectable culturable cells after exposure for 26 h (Fig. 1B), even after concentration by filtration. Plate counts of cells incubated in the dark did not decrease (Fig. 1C). However, as soon as exposure of these cells to light occurred, the plate counts decreased, but in both cases, the AODC and DVC did not change significantly. Plate counts of samples of surface water in chambers suspended in the Morlaix Estuary on 20 June 1988 showed a rapid decrease with exposure to sunlight (Fig. 2B), whereas plate counts for samples from chambers incubated at the bottom remained stable, i.e., the number of culturable bacteria did not change significantly (Fig. 2C). Near the surface, during these experiments, no significant difference was found between quartz and plexiglass chamber counts, i.e., in the effect of UV or visible light.

Light intensity during the day was significant, both in June and October (>1,000 $\mu Em^{-2}S^{-1}$), for surface samples. In the field, as previously reported (17), light intensity just above the bottom was significantly less (<100 $\mu Em^{-2}S^{-1}$), with suspended and/or dissolved organic matter contributing to light absorption and, therefore, stability of the plate counts because of decreased light penetration.

Rabbit intestinal loops. Results of analyses of fluid accumulation in the rabbit loops are given in Table 1. Fluid accumulation associated with seawater was sufficiently extensive that it masked toxigenic activity of the cells, a phenomenon reported by Johnson and Calia (12) to occur in experiments carried out using *Vibrio parahaemolyticus* suspended in seawater.

GM1-ELISA. Fluid in loops of the rabbit (R2) was examined by GM1-ELISA, as described in the Materials and Methods, and results are given in Table 2. In the direct assays, samples 2, 3, and 4 were tested after the *E. coli* strain had been incubated in the loops for 7 h, yielding positive results in the GM1-ELISA, whereas the sterile sample (sample 1) was negative. Assays done after the loop contents had been cultured in MCA broth for 24 h at 37°C, with shaking, showed only sample 2 yielding a positive result.

The seawater control (sample 1) produced very slight but measurable fluid accumulation in rabbit intestinal loops. In the study reported here, interloop leakage contamination did not occur and was not, therefore, responsible for the observed fluid accumulation in the loops. Indeed, examination by GM1-ELISA of the interloops immediately upon inoculation and after inoculation revealed the loops were negative for *E. coli* H10407. Thus, the effect of seawater is concluded to account for the very slight accumulation of fluid (12), although temperature may also have had an effect (see below).

E. coli H10407, after exposure to seawater, i.e., samples 2 (culturable) and 3 (nonculturable), was able to produce toxin (LT) in rabbit intestinal loops, as confirmed by GM1-ELISA (Table 2). Thus, it is concluded that *E. coli* exposed to seawater retains the ability to produce enterotoxin, whether culturable or nonculturable, a phenomenon observed also for *Shigella dysenteriae* type 1 (19).

Whether nonculturable *E. coli* cells producing enterotoxin represent only a few active cells or a total population of slowly metabolizing cells was addressed as follows. If a few actively metabolizing cells were present in the sample, they should have been recoverable on Drigalski's medium, but none was recovered. The sample was filtered, using a 0.22-μm filter, before incubation to concentrate the cells before testing for growth. Roszak and Colwell (20), using radioautography, demonstrated that metabolic activity can be observed for cells incubated with radiolabeled substrate and such activity was not the response of only a few actively metabolizing cells among a viable but nonculturable population but, instead, that of the majority of cells. Similarly, in this study, the DVC data support

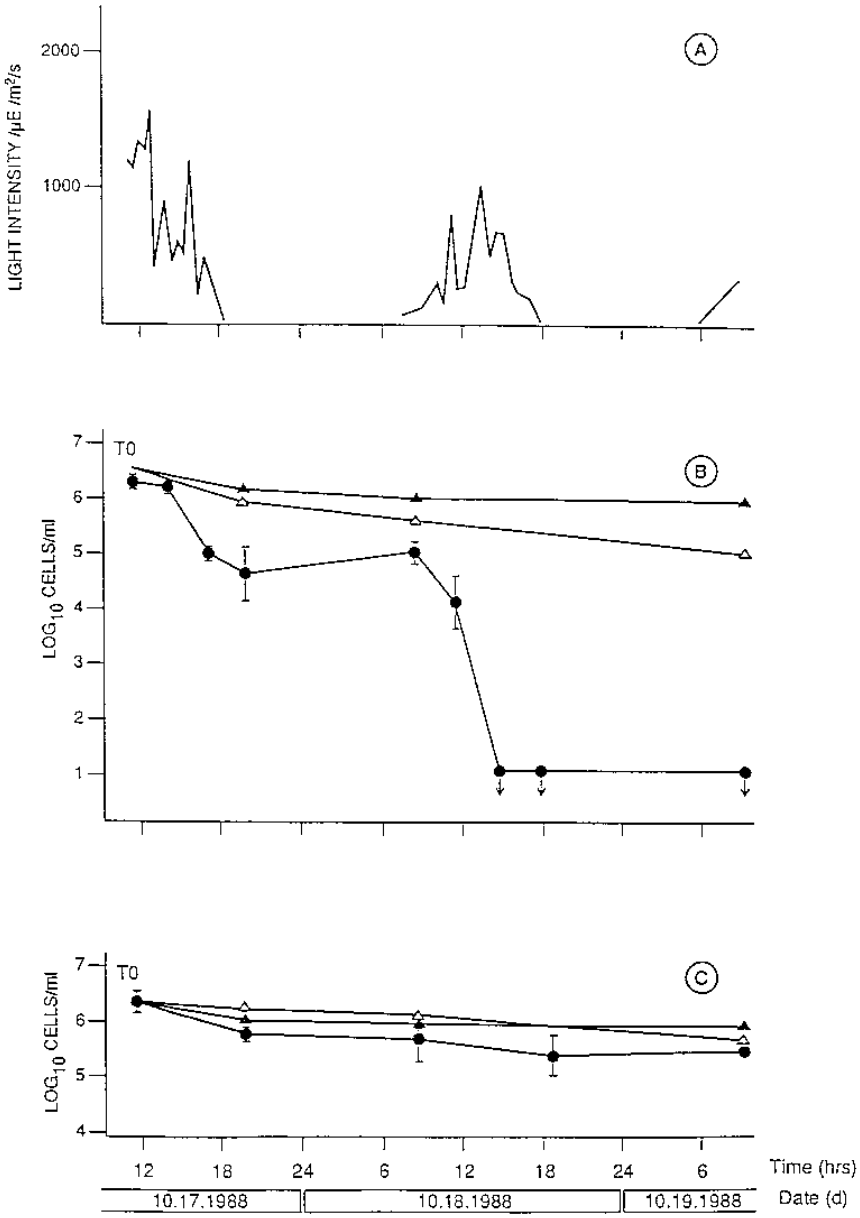

FIG. 1. Effect of solar radiation on *E. coli* H10407 in filtered surface seawater. (A) Light intensity. AODC (▲), DVC (△), and plate counts (●) were calculated for samples collected from flasks incubated in the light (B) or flasks incubated in the dark (C). Standard deviations are indicated by bars.

the interpretation that the bulk of the cells were metabolically active and, therefore, capable of producing enterotoxin, as is the case for *S. dysenteriae* type 1 (19).

It is important to note that the intestinal flora of the rabbit prior to preparation of loops and inoculation with *E. coli* H10407 was tested and found negative for enterotoxigenic *E. coli*. Only toxin produced by the inoculated cells was detected by GM1-ELISA. Furthermore, in the case of sample 3, the loop fluid tested by GM1-ELISA was positive for toxin, indicating that *E. coli* in the viable but nonculturable state retained pathogenicity. However, *E. coli* H10407 in the viable but nonculturable state did not revert to culturability in numbers sufficient to dominate the culturable intestinal flora after incubation for 7 h, i.e., to be detectable by plating on selective medium. A similar phenomenon was observed for *V. cholerae*, namely, that the presence of *V. cholerae* 01 was most effectively demonstrated by immunofluorescence staining of loop contents (10). A saline suspension of *E. coli* H10407 cells (sample 4) incubated for 3 days at 20°C, a lower temperature employed to approximate more closely the environmental conditions of the Morlaix Estuary, produced detectable LT, but little fluid accumulation. Similar results were observed by Singh et al. (23) for cells suspended in drinking water. However, in this study, toxin was produced by nonculturable *E. coli* cells suspended in physiological saline after the cell suspension had been incubated for 3 days at 20°C.

On the basis of these results and those of earlier studies (10), it is concluded that enteropathogenic *E. coli* discharged into seawater in estuaries and coastal waters can retain enteropathogenicity. Since results of studies employing *Helicobacter pylori* indicate that cells of *H. pylori* exposed to river water in laboratory microcosms for up to 2 years can retain viability and

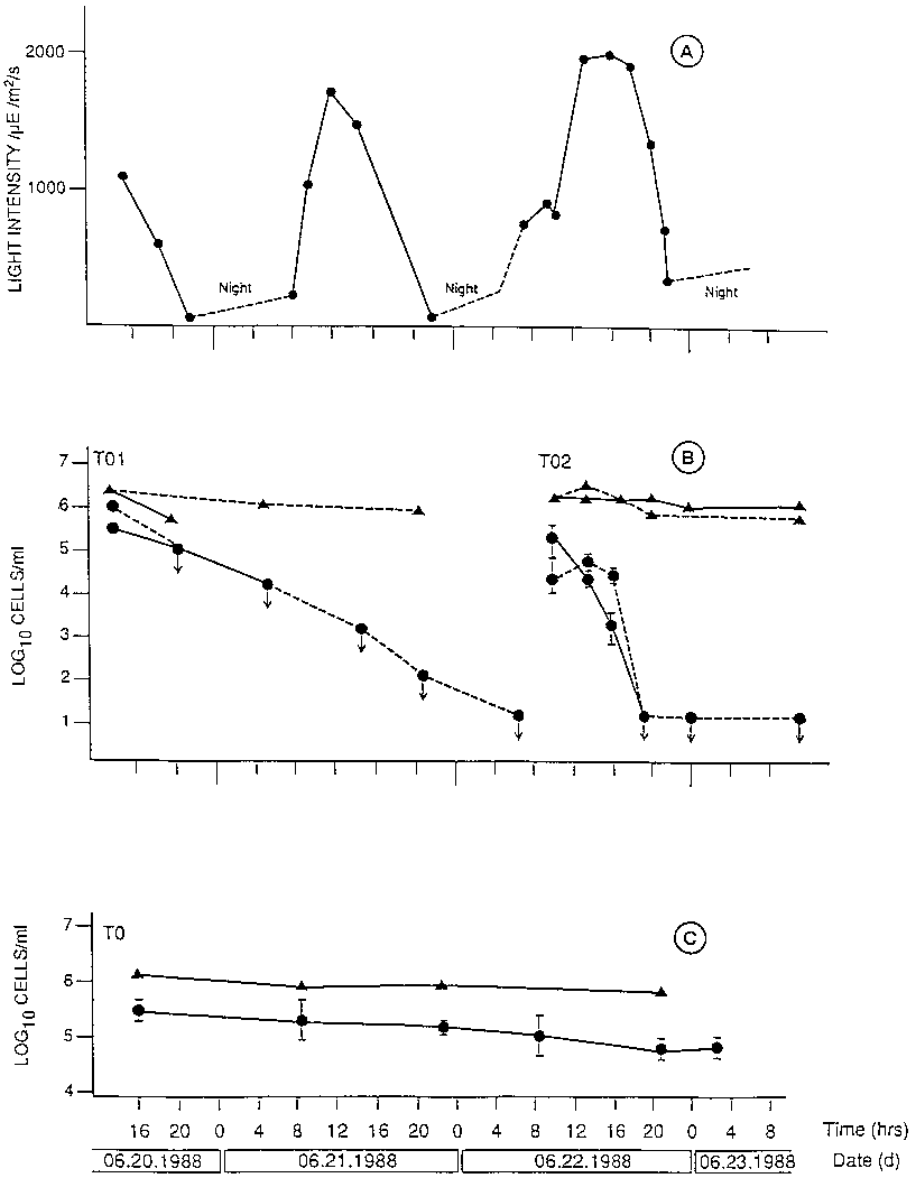

FIG. 2. Effect of solar irradiation on *E. coli* H10407 in membrane chambers. In the Bay of Morlaix light intensity was measured just below the surface (A), in surface chambers (B), and in bottom chambers (C). AODC (▲) and plate counts (●) were calculated for samples collected from quartz (−) or plexiglass (– – –) chambers. Standard deviations are indicated by bars.

selected properties associated with pathogenesis (22), the phenomenon reported here is concluded not to be associated only with *E. coli*. Furthermore, the results reported here are compatible with the findings of Barcina et al. (1) and Davies and Evison (6), who carried out laboratory experiments with natural and artificial light. In their studies, field experiments were done in parallel with laboratory experiments to demonstrate that the laboratory experiments showing effects of exposure of

TABLE 1. Fluid accumulation in rabbit loops[a]

Sample	Treatment	Loop no. (sequence)[b]	Fluid accumulation	Loop	Fluid accumulation
1	Seawater without *E. coli*	3	0.91 (weak +)	2	0.91 (weak +)
2	*E. coli* H10407 LT^+ in bottom seawater chamber	4	0.87 (weak +)	1	1.20 (+)
3	*E. coli* H10407 LT^+ in surface seawater chamber	1	0.84 (weak +)	3	0.76 (weak +)
4	*E. coli* H10407 LT^+ incubated for 3 days in physiological saline at 20°C	2	0.43 (−)	4	0.51 (weak +)

[a] Fluid accumulation is the ratio of weight (grams)/length (centimeters).
[b] Loop number 1 was located just below the stomach.

TABLE 2. Detection of toxin by GM1-ELISA[a]

Sample no.	Treatment	Fluid in loop after 7 h	Fluid in loop after inoculation of loop contents into GM1-ELISA broth and incubation for 24 h at 37°C
1	Seawater without *E. coli*	0.034−	0.000−
2	*E. coli* H10407 LT^+ in bottom seawater chamber	1.310+	>2.000+
3	*E. coli* H10407 LT^+ in surface seawater chamber	0.139+	0.000−
4	*E. coli* H10407 LT^+ incubated for 3 days at 20°C in physiological saline	0.234+	ND[b]
E. coli HB101 LT^-	NA[c]	NA	0.029
E. coli H10407 LT^+	NA	NA	>2.000+

[a] Values represent optical density (OD) readings for each sample after subtraction of OD in Mundell's Casamino Acids medium. Positive reaction threshold was estimated as 0.100.
[b] ND, not determined.
[c] NA, not applicable, i.e., control.

E. coli H10407 to natural sunlight in seawater and conversion of cells to the viable but nonculturable state, with retention of enterotoxigenicity, were the same as those obtained in situ, i.e., as would occur upon discharge to an estuary of run-off or sewage containing enterotoxigenic *E. coli*. The die-off ascribed to natural sunlight (4) more appropriately may be viewed as inducing the viable but nonculturable state in many, if not all, cells, rather than the die-off or death of all cells.

Bacteriological methods employed in the analysis of public health safety of natural waters, especially seawater, must be reassessed if potentially pathogenic bacteria can not only survive but retain enterotoxigenicity when not detectable by standard bacteriological culture methods. In addition, accumulation of viable but nonculturable *E. coli* H10407 by shellfish in such waters may pose a potentially serious problem, a possibility that merits further study. It has frequently been observed that shellfish can contain high fecal coliform counts when the surrounding seawater is sufficiently low in coliform count to be considered safe for shellfish harvesting. Halotolerance of *E. coli* and reduction of light penetration when particulate matter is present have been shown to be responsible for *E. coli* continuing to be culturable for days, weeks, or longer (17, 18, 21). When ingested by shellfish, the *E. coli* and related bacteria may retain culturability when the same bacteria in the surrounding seawater exposed to sunlight may be viable but nonculturable, leading to a disparity between the shellfish and water counts. In light of these considerations, the die-off calculation employed by sanitary engineers for measuring the quality of seawater at sewage outfalls may be too simple a measurement, at least in the context of public health. At the minimum, a reevaluation of assumptions made in such calculations is needed and detection methods, other than culturing, are needed for accurate measurement of the potential pathogens and/or indicator organisms that may be present.

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REFERENCES

1. **Barcina, I., J. M. Gonzalez, J. Iriberri, and L. Egea.** 1990. Survival strategy of *Escherichia coli* and *Enterococcus faccalis* in illuminated fresh and marine systems. J. Appl. Bacteriol. **68:**189–198.
2. **Bothner, M. H., H. Takda, I. T. Knight, R. T. Hill, B. Butman, J. W. Farrington, R. R. Colwell, and J. F. Grassle.** 1992. Sewage contamination in sediments beneath a deep-ocean dump site off New York. Marine Environ. Res. **38:**43–59.
3. **Byrd, J. J., and R. R. Colwell.** 1990. Maintenance of plasmids pBA 322 and pUC 8 in noncultivable *Escherichia coli* in the marine environment. Appl. Environ. Microbiol. **56:**2104–2107.
4. **Chamberlin, C. E., and R. Mitchell.** 1978. A decay model for enteric bacteria in natural waters, p. 325–368. *In* R. Mitchell (ed.), Water pollution microbiology, vol. 2. Wiley, New York.
5. **Colwell, R. R., P. R. Brayton, D. J. Grimes, D. R. Roszak, S. A. Huq, and L. M. Palmer.** 1985. Viable, but nonculturable *Vibrio cholerae* and related pathogens in the environment: implications for release of genetically engineered microorganisms. BioTechnology **3:**817–820.
6. **Davies, C. M., and L. M. Evison.** 1991. Sunlight and the survival of enteric bacteria in natural waters. J. Appl. Bacteriol. **70:**265–274.
7. **Desmonts, C., J. Minet, R. R. Colwell, and M. Cormier.** 1990. Fluorescent antibody method useful for detecting viable but nonculturable *Salmonella* spp. in chlorinated waste water. Appl. Environ. Microbiol. **56:**1448–1452.
8. **Evans, D. G., R. P. Silver, D. J. Evans, Jr., D. G. Chase, and S. L. Gozbach.** 1975. Plasmid-controlled colonization factor associated with virulence in *Escherichia coli* enterotoxigenic for humans. Infect. Immun. **12:**656–667.
9. **Fliermans, C. B., and R. W. Gorden.** 1977. Modification of membrane diffusion chambers for deep water studies. Appl. Environ. Microbiol. **33:**207–210.
10. **Grimes, D. J., R. W. Attwell, P. R. Brayton, L. M. Palmer, D. M. Rollins, D. B. Roszak, F. L. Singleton, M. L. Tamplin, and R. R. Colwell.** 1986. The fate of enteric pathogenic bacteria in the estuarine and marine environment. Microbiol. Sci. **3:**326–329.
11. **Hobbie, J. E., R. J. Daley, and S. Jasper.** 1977. Use of nuclepore filters for counting bacteria by fluorescence microscopy. Appl. Environ. Microbiol. **33:** 1225–1228.
12. **Johnson, D. E., and F. M. Calia.** 1976. False positive rabbit ileal loop reactions attributed to *Vibrio parahaemolyticus* broth filtrate. J. Infect. Dis. **33:**436.
13. **Knight, I. T., S. Shults, C. W. Kaspar, and R. R. Colwell.** 1990. Direct detection of *Salmonella* spp. in estuaries by using a DNA probe. Appl. Environ. Microbiol. **56:**1059–1066.
14. **Kogure, K., U. Simidu, and N. Taga.** 1979. A tentative direct microscopic method for counting living marine bacteria. Can. J. Microbiol. **25:**415–420.
15. **LaBelle, R. L., and C. P. Gerba.** 1980. Influence of estuarine sediment on virus survival under field conditions. Appl. Environ. Microbiol. **39:**749–755.
16. **Mundell, D. H., C. R. Antelmo, and R. M. Wishnow.** 1976. Factors influencing heat-labile *Escherichia coli* enterotoxin activity. Infect. Immun. **16:**383–388.
17. **Pommepuy, M., J. F. Guillaud, Y. Martin, E. Dupray, A. Derrien, J. L'Yavanc, and M. Cormier.** 1990. Le devenir des bactéries en zone littorale. La Mer et les Rejets Urbains. Bandor, 13–15, June, 1990. IFREMER, Actes de Colloques. **11:**89–100.
18. **Pommepuy, M., J. F. Guillaud, E. Dupray, A. Derrien, F. LeGuyadei, and M. Cormier.** 1992. Enteric bacteria survival factors. Water Sci. Technol. **12:**93–103.
19. **Rahman, I., M. Shahamat, M. A. R. Chowdhury, and R. R. Colwell.** 1996. Potential virulence of viable nonculturable *Shigella dysenteriae* type 1. Appl. Environ Microbiol. **62:**115–120.
20. **Roszak, D. B., and R. R. Colwell.** 1987. Metabolic activity of bacterial cells enumerated by direct viable count. Appl. Environ. Microbiol. **53:**2889–2893.
21. **Salomon, J. C., and M. Pommepuy.** 1990. Mathematical model of bacterial contamination of the Morlaix Estuary (France). Water Res. **24:**983–996.
22. **Shahamat, M. C., C. Paszko-Kolva, U. E. Mai, H. Yamamoto, and R. R. Colwell.** 1992. Selected cryopreservatives for long term storage of *Helicobacter pylori* at low temperatures. J. Clin. Pathol. **45:**735–773.
23. **Singh, A. R., R. Yeager, and G. A. McFeters.** 1986. Assessment of in vivo revival, growth, and pathogenicity of *Escherichia coli* strains after copper and

chlorine-induced injury. Appl. Environ. Microbiol. **52:**832–837.
24. **Singleton, F. L., R. W. Attwell, M. S. Jangi, and R. R. Colwell.** 1982. Influence of salinity and organic nutrient concentration on survival and growth of *Vibrio cholerae* in aquatic microcosms. Appl. Environ. Microbiol. **43:**1080–1085.
25. **Svennerholm, A. M., M. Wikaström, M. Luidblad, and J. Holmgren.** 1986. Monoclonal antibodies to *Escherichia coli* heat-labile enterotoxins: neutralizing activity and differentiation of human and toxigenic LTs and cholera toxin. Med. Biol. **64:**23–30.
26. **Xu, H. S., N. Roberts, F. L. Singleton, R. W. Attwell, P. J. Grimes, and R. R. Colwell.** 1982. Survival and viability of nonculturable *Escherichia coli* and *Vibrio cholerae* in the estuarine and marine environment. Microb. Ecol. **8:**313–323.