

In Situ Development of Sublethal Stress in *Escherichia coli*: Effects on Enumeration†

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Development of sublethal stress in *Escherichia coli* exposed in situ to estuarine waters was examined during various seasons. An electrochemical detection technique was utilized to derive a stress index based upon the difference between a predicted electrochemical response time in Trypticase soy broth or EC medium at 44.5°C estimated from a standard curve for unstressed cells and an observed response time for cells exposed to seawater. This stress index was related to recovery efficiencies of seawater-exposed cells, using a variety of standard and resuscitative enumeration procedures. Stress was further studied by determination of the adenylate energy charge. Sublethal stress as measured by the electrochemical detection method was an inverse function of water temperature, with maximum stress occurring after exposure to temperatures below 10°C. Total adenylates and ATP decreased dramatically at low temperatures, although energy charge remained relatively constant under various environmental conditions. Decreases in *E. coli* ATP suggest that ATP may not be an adequate measure of biomass for in situ stressed cells. Discrepancies in enumeration efficiency were most pronounced at temperatures below 10°C. Resuscitative procedures for solid-media techniques increased the recovery of stressed cells under cold water conditions but were not as effective as the standard most-probable-number procedure.

The existence of sublethal injury in populations of enteric bacteria exposed to aquatic environments is well documented (2, 5, 7, 15, 20, 27). Surviving bacteria may exhibit injury to structural or functional components (or both) related to metabolic activities and be rendered sensitive to previously uninjurious chemical compounds and temperatures (22). Since specific enumeration procedures frequently incorporate stressors of both selective media and elevated incubation temperatures, that portion of sample populations containing sublethally injured cells may not be detected. Factors leading to underestimation of bacterial indicator groups utilized as indices of sanitary water quality are of obvious concern.

Significant differences between most-probable-number (MPN) and membrane filter enumerations of fecal coliforms from estuarine and marine water samples have been attributed to sublethal stress. Investigators utilizing the standard M-FC procedure (1) have reported that membrane filter counts enumerated less than 50% of the fecal coliforms recovered by the

standard MPN method (21, 25). Presumably, the MPN procedure allowed for repair during nonselective enrichment in liquid presumptive media. Recent developments have focused on modifying conventional solid media procedures to allow for a similar repair phase for stressed cells. Approaches to improve recovery of fecal coliforms have included delayed exposure to the selective temperature of 44.5°C (8, 9) as well as delayed exposure to selective media (10, 23, 25). Application of resuscitation procedures to membrane filtration and plating techniques have yielded results in closer agreement with MPN estimates (9, 10, 25).

Most studies evaluating recovery methods for fecal coliforms exposed to seawater have been limited to comparative enumerations of environmental grab samples without specific regard to the effects of exposure duration, fluctuations in physicochemical conditions, or both. Therefore, the objective of our present research was to assess the development of sublethal stress in fecal isolates of *Escherichia coli* after in situ estuarine exposure during various seasons and to determine the influence of stress on various enumeration procedures. Enumeration techniques evaluated included the standard MPN

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and membrane filter procedures (1) for determination of fecal coliforms in seawater and two proposed methods (10, 23) for improved recovery of fecal coliforms from marine samples. An electrochemical detection technique which measures the time required for a bacterial inoculum to produce an increase in electric potential in culture media had previously been reported (2) as a technique for studying sublethal stress in *E. coli*. Cells which had been exposed in vitro to seawater of various salinities produced delayed response times, the magnitude of which was directly proportional to salinity. In the present study, the electrochemical detection technique was employed to assess the effects of exposure time under various environmental water temperature regimes. Adenylate energy charge was also determined as an expression of in situ development of stress.

MATERIALS AND METHODS

Chamber design and construction. McFeters-Stuart diffusion chambers (17) were modified to accommodate an increased volume of test suspension and to minimize previously observed contamination by indigenous microorganisms in seawater, presumably through leakage between membrane-chamber interfaces. Chambers were fabricated from Tuffak (Rohm & Haas Co., Philadelphia, Pa.), a polycarbonate plastic capable of withstanding repeated autoclaving. Chamber design included a central spacer constructed of 1/2-in. (1.27 cm)-thick Tuffak with outer retainer plates of 1/4-in. (0.635 cm) Tuffak. This design formed a chamber volume of 40 ml by apposition of the central spacer (57-mm opening) with two 73-mm-diameter polycarbonate membranes (0.2 μ m; Nuclepore Corp., Pleasanton, Calif.) held in place by the two retainer plates secured with six stainless steel bolts and nuts. To prevent leakage and contamination, 70-mm O-ring grooves were machined into the inside faces of the retainer plates and fitted with 1/4-in. (0.635-cm)-thick Buna-N O-rings. Two sampling ports were drilled in the central spacer, and they were fitted with serum bottle stoppers (7/32 in. [ca. 0.56 cm], no. 7116-00; Bittner Corp., Norcross, Ga.) and secured with steel-filled epoxy.

Assembly of chamber. Serum stoppers were installed and the epoxy was allowed to cure for 24 h. Autoclaved polycarbonate chamber parts and UV-irradiated membranes were assembled aseptically. Lubriseal (Arthur Thomas Co., Philadelphia, Pa.) was used to hold the membranes against the central spacer during assembly.

Organisms. Fecal coliforms were freshly isolated from human feces by enrichment in lactose broth (BBL Microbiology Systems, Cockeysville, Md.) at 35°C for 24 h followed by inoculation into EC broth (BBL) at 44.5°C for 24 h. Isolates producing gas in EC were streaked onto eosin methylene blue agar (BBL) and incubated at 35°C for 24 h. A typical green sheen colony was streaked onto Trypticase soy agar (TSA, BBL) for identification with an API 20E (Analytab Products, Inc., Plainview, N.Y.) test strip. Isolates were maintained on TSA slants at 4°C and discarded after 6 weeks.

Preparation of chamber inocula. *E. coli* cultures were either grown in M9 medium (pH 7.0) (19) supplemented with 0.5% glycerol instead of glucose or in Trypticase soy broth (TSB). Cultures were incubated at 35°C for 12 h, diluted 500-fold into fresh medium, and incubated an additional 12 h.

Seawater exposure studies. Duplicate diffusion chambers were inoculated with a suspension containing approximately 10^7 to 10^8 bacteria per ml in twice-membrane-filtered (0.45 and 0.2 μ m) seawater. Chambers were immediately deployed in the York River, Va., in water of approximately 1.5-m depth at low tide. The chambers, in protective wire baskets 1 m below the surface, were suspended from floats tethered to a pier. During the summer it was necessary to place a layer of nylon mesh over the chambers to protect them from barnacles, tunicates, and other fouling organisms. Samples were removed from chambers at selected intervals with sterile hypodermic syringes equipped with 20-gauge needles. Before sampling, membranes were inspected for damage, chambers were shaken, and port plugs were swabbed with 95% alcohol and flamed.

Enumeration techniques. Recovery of *E. coli* by using selective enumeration methods was always compared to recovery on TSA. Enumeration on TSA was performed by spread plating and incubation at 35 \pm 0.5°C for 24 \pm 2 h. Selected enumeration procedures were: (i) five-tube MPN technique (1), using lactose broth at 35 \pm 0.5°C for 48 \pm 3 h with confirmation in EC broth at 44.5 \pm 0.2°C for 24 \pm 2 h; (ii) the M-FC procedure (1) with incubation in a heat block incubator (Millipore Corp., Bedford, Mass.) at 44.5 \pm 0.2°C for 24 \pm 2 h; (iii) the M-FC method as modified by Rose et al. (23), using M-FC agar overlaid with lactose agar with incubation at 35 \pm 0.5°C for 2 h before transfer to a heat block incubator at 44.5 \pm 0.2°C for 22 \pm 2 h; (iv) spread plating onto violet red bile agar (VRBA) with immediate incubation in an air incubator at 44.5 \pm 0.5°C for 24 \pm 2 h; and (v) the repair method of Hackney et al. (10), modified to include spread plating onto TSA with incubation at room temperature for 2 h followed by addition of 10 ml of VRBA overlay and incubation at 44.5 \pm 0.5°C for 22 \pm 2 h. Duplicate plates were prepared for each dilution.

Determination of EDT. Instrumentation for determining electrochemical detection time (EDT) has previously been described (2). During in situ exposure, samples were removed at timed intervals for plate count enumeration on TSA at 35°C and EDT determination. Two milliliters of chamber contents was inoculated into 18 ml of EC medium or TSB prewarmed in a water bath at 44.5 \pm 0.2°C, and the time elapsed between inoculation and the maximum change in potential was measured. Stress was defined and plotted as the difference between the predicted EDT calculated for nonexposed cells from a standard curve and the observed EDT for cells after exposure to estuarine water. Standard curves relating inoculation size to EDT were established for each isolate, using a linear least-squares regression technique. The 95% confidence limits were determined by calculating the standard error of the estimated EDT from the linear regression (24).

Determination of adenylate energy charge ratios. Adenylates were extracted and assayed by the basic procedure of Karl and Holm-Hansen (14) with several

modifications for measurement of low concentrations of adenine nucleotides. A 1-ml portion of cell suspension was extracted in 5 ml of boiling Tris buffer (0.02 M, pH 7.7) for 5 min. A 1-ml portion of cell-free medium (0.45- μ m filtrate) was similarly treated, and intracellular adenine nucleotide levels were determined as the difference between adenylate concentrations in the complete culture and in the cell-free filtrate. Firefly luciferin-luciferase enzyme mixture (FLE-50; Sigma Chemical Co., St. Louis, Mo.) was aged in 5 ml of distilled water in the dark at room temperature for 24 h and centrifuged ($<2,000 \times g$) for 10 min to sediment particulates. Ten milliliters of Tris buffer and 10 ml of $MgSO_4$ (0.04 M) were then added to the removed supernatant, and the enzyme mixture was iced during the assay procedure. Potassium phosphate buffer (0.12 M K_2HPO_4 and 0.03 M KH_2PO_4) and $MgSO_4$ (0.03 M) were substituted for sodium phosphate buffer and $MgCl_2$ in assay tubes A, B, and C. The bioluminescence reaction was initiated by adding 0.5 ml of enzyme solution to 0.2 ml of sample extract or standard solution. Emitted light was measured by using an SAI integrating photometer (model 2000; Science Applications, Inc., La Jolla, Calif.).

Sodium salts of ATP, ADP, and AMP, the trisodium salt of phosphoenolpyruvate, pyruvate kinase, and myokinase were purchased from Sigma.

All samples and standards were run in duplicate. Standards were assayed before and after sample analyses. Adenylate concentrations were determined from linear regressions of appropriate standard curves. Energy charge was calculated (4) as $[ATP + 1/2 (ADP)] / (ATP + ADP + AMP)$.

RESULTS

EDT stress index. Sublethal stress measured by the electrochemical detection method was inversely related to water temperature (Fig. 1 and 2). Maximum delays in EDT occurred at temperatures below 10°C and tended to progressively increase with exposure time. Stress was manifested to a greater extent in the selective EC medium than in TSB (Fig. 1). During one series of experiments, the 0-h sampling was performed 5 min after mixing bacteria with seawater at ambient river temperature (Fig. 2) instead of mixing with seawater equilibrated to room temperature (Fig. 1). Under these conditions, test suspensions demonstrated an immediate sensitivity to low temperatures as manifested by an approximately 3-h delay in EDT.

Energy charge stress index. The results of

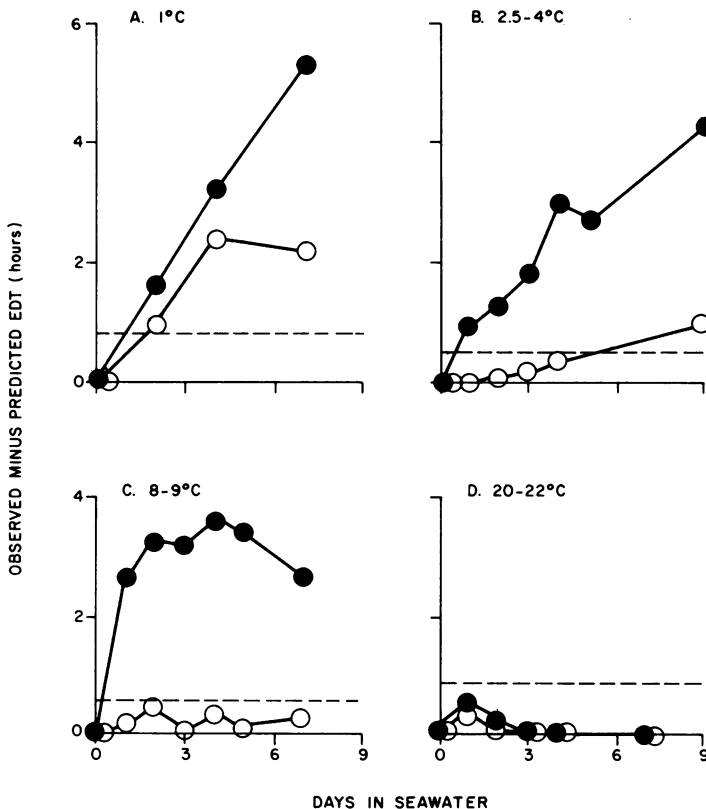


FIG. 1. Incidence of stress as measured by the electrochemical detection method for *E. coli* pregrown in M9 and exposed to seawater at various temperatures. EDT determined with TSB (○) and EC (●) at 44.5°C. Upper 95% confidence limit for predicted EDT indicated as ----. Mean salinity for each experiment was 24 ppt.

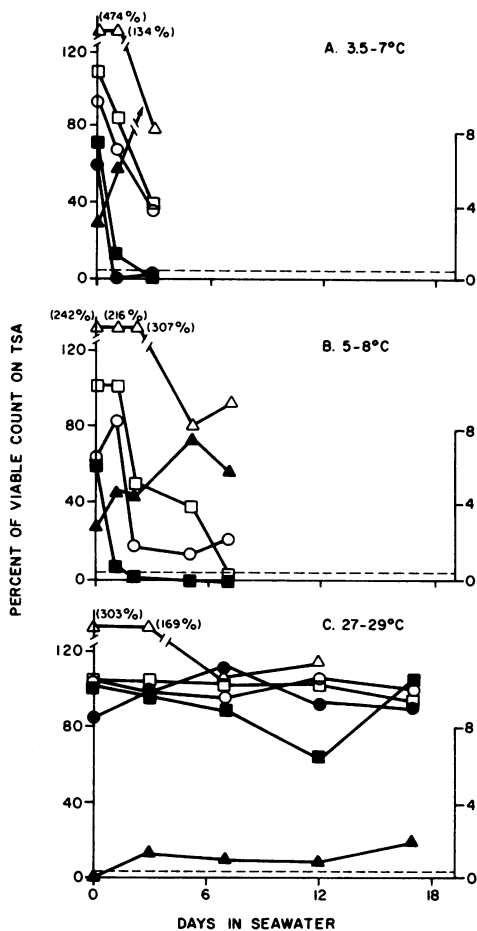


FIG. 2. Incidence of stress as measured by enumeration efficiency and the electrochemical detection method for *E. coli* pregrown in M9 and exposed to seawater at various temperatures. Enumeration efficiency calculated as percent viable count on TSA. EDT determined with EC at 44.5°C. Upper 95% confidence limit for predicted EDT indicated as ----. Mean salinities for experiments A, B, and C were 18, 18.5, and 21 ppt, respectively. Symbols: Δ , MPN; \circ , VRBA overlay; \bullet , VRBA direct; \square , M-FC overlay; \blacksquare , M-FC direct; \blacktriangle , EDT.

energy charge determinations for laboratory batch-grown cultures and cells after in situ exposure are shown in Fig. 3. Before environmental exposure, viable cell counts and concentrations of ATP and total adenylates increased. Upon exposure to 4.5 and 9°C waters, ATP and total adenylates decreased more rapidly than did the viable count. In contrast, cells exposed to 27°C showed increased viable counts and increased ATP and total adenylates during the first 2 days of exposure followed by a slow decline in ATP and total adenylates. Although viable counts and adenylate concentrations varied widely depend-

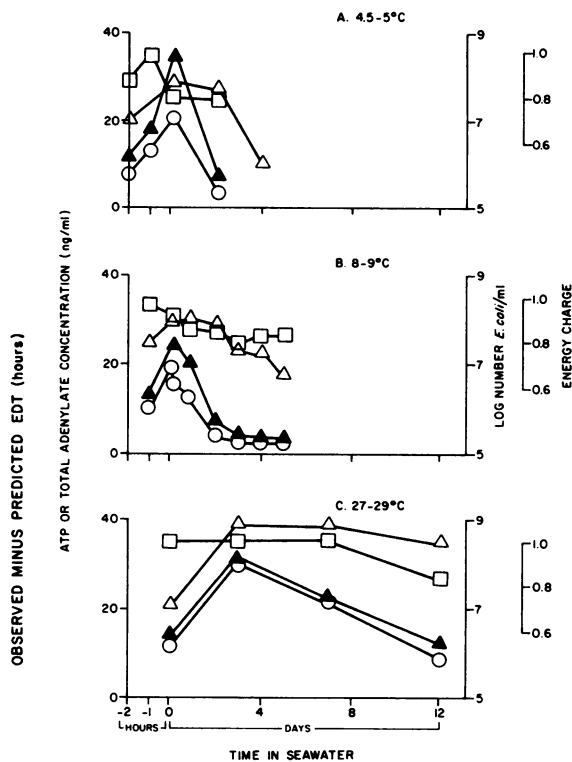


FIG. 3. Incidence of stress as measured by energy charge, ATP, and total adenylates for *E. coli* pregrown in M9 and exposed to seawater at various temperatures. Mean salinities for experiments A, B, and C were 17, 24, and 20 ppt, respectively. Symbols: \square , ATP; \circ , energy charge; \bullet , total adenylates; and Δ , log number of *E. coli* per ml.

ing upon the environmental conditions encountered, energy charge appeared to remain relatively stable. However, rapid loss of ATP from cells exposed to cold water complicated energy charge determinations. By using crude luciferin-luciferase extracts, the lowest concentration of ATP that could be accurately assayed in these experiments was approximately 5 ng/ml. Addition of exogenous luciferin to the reactive mixture (12) did not improve sensitivity. Thus, when cells were most stressed, as defined by decreasing ATP and adenylate values, it was not possible to accurately determine energy charge.

Comparative enumeration efficiency. Recovery efficiencies of standard and proposed fecal coliform enumeration methods were determined for pure cultures of *E. coli* exposed to estuarine waters during different seasons (Fig. 2). Percent recovery was calculated relative to cell counts obtained on TSA. Zero-hour determinations revealed that enumeration discrepancies were evident immediately after exposure to cold seawater.

ter. Direct application of selective media and temperature in the M-FC and VRBA plating procedures resulted in dramatic decreases in *E. coli* counts. Resuscitation improved recovery but modified plating methods detected fewer than 40% of the viable population after 3 days at 3.5°C or 5 days at 6°C. The MPN method appeared the most effective procedure for recovery of stressed cells from cold water, with estimates up to threefold higher than plate counts on TSA. With direct selective plating, bacterial enumerations were below the lower 95% confidence limit of MPN values after a 1-day exposure to 3.5 or 6°C water. VRBA and M-FC overlay techniques resulted in plate counts which fell within the 95% confidence limits of the MPN values for the 3.5°C experiment. However, when a similar experiment at 6°C was extended, enumerations by both overlay techniques fell below the lower MPN confidence limits between 2 and 7 days. Application of a correction factor of $e^{-0.805/N}$, where N equals the number of replicate tubes to correct for the positive bias inherent in the MPN (16) procedure, did not significantly alter the relative recovery efficiency of the MPN method.

As water temperatures increased, repair procedures provided better recovery. The modified VRBA and M-FC overlay procedures recovered 98 and 78%, respectively, of the viable population after a 9-day exposure to 16 to 18.5°C seawater (not graphically presented). When the estuary warmed to 27°C, *E. coli* was effectively recovered by all selective procedures.

Effect of Pretreatment. All preceding experiments were conducted with unwashed cells grown in a minimal medium to early-stationary phase. This pretreatment regime was selected to minimize sublethal stress. To determine whether more complex manipulations influenced the development of stress during in situ exposure, inocula were grown in a rich medium (TSB), harvested by centrifugation, washed three times, and refrigerated for 6 h according to a previous method used for survival studies of enteric bacteria in seawater (28). Under these conditions, *E. coli* suspensions exposed in situ to 28°C water showed increased sublethal stress as measured by the electrochemical detection method (Fig. 4), decreased recovery efficiency by all enumeration techniques (Fig. 5), depressed energy charge, and low initial concentrations of ATP and total adenylates (Fig. 6) compared with unwashed cultures grown in minimal medium (Fig. 2C and 3C).

DISCUSSION

The present study demonstrated a correspondence between sublethal injury and relative enumeration efficiency for *E. coli* exposed to estua-

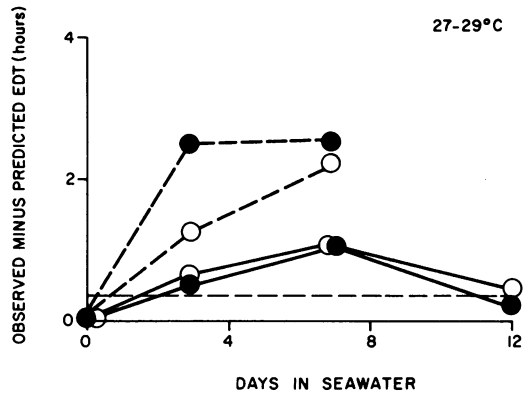


FIG. 4. Effect of pretreatment on incidence of stress as measured by the electrochemical detection method for *E. coli* exposed to 27°C, 20-ppt-salinity seawater. EDT determined with TSB (○) and EC (●) at 44.5°C. Cells pregrown in M9 (—) or TSB (---). Upper 95% confidence limit for predicted EDT indicated as horizontal ----.

rine waters. The extent of sublethal injury sustained was directly related to time and inversely related to water temperature. Harsh laboratory manipulations employed before in situ exposure intensified debilitation.

Sublethal injury in *E. coli* has been observed during exposure to low temperatures in freshwater (5) and seawater (7) environments as well as in frozen foods (22). Various resuscitation techniques have been designed to recover damaged cells by providing nutrients and favorable temperatures to effect repair before exposure to selective conditions. However, attention has not been directed to the expression of sublethal stress as a function of exposure time or seasonal conditions (temperature) or to the effect of stress on enumeration.

Results presented here demonstrate that increased stress resulted in corresponding reductions in relative enumeration efficiency. Since debilitation within a population was progressive at low temperatures, the comparative efficiencies of various techniques changed during continued in situ exposure. Although resuscitative enumeration procedures such as the modified M-FC or VRBA overlay techniques effectively recovered more injured fecal coliforms than the standard M-FC or VRBA procedures, none of the solid-media repair techniques recovered as many cells stressed under cold water conditions as the standard fecal coliform MPN method. Maximum recovery with the latter procedure was probably attributable to the resuscitation of injured cells in a nonselective medium during the presumptive test phase before exposure to the selective medium at an elevated incubation temperature. However, estimates provided by the

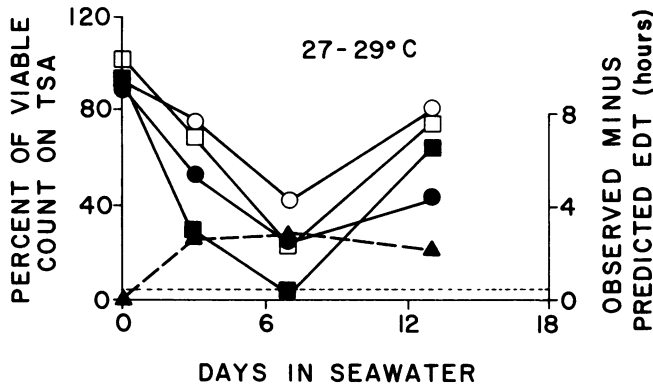


FIG. 5. Incidence of stress as measured by enumeration efficiency and the electrochemical detection method for *E. coli* pregrown in TSB and exposed to 27°C, 20-ppt-salinity seawater. Enumeration efficiency presented as percent viable count on TSA. EDT determined in EC at 44.5°C. Upper 95% confidence limit for predicted EDT indicated as ----. Symbols: ○, VRBA overlay; ●, VRBA direct; □, M-FC overlay; ■, M-FC direct; ▲, EDT.

MPN method are positively biased, less precise, and more time-consuming than plate count procedures.

The effectiveness of proposed resuscitative procedures for plate count enumerations of stressed fecal coliforms in environmental samples has been a topic of considerable interest. The modified M-FC method was originally evaluated against the standard M-FC procedure for recovery of fecal coliforms from various aquatic environments (23) but included only a limited number of samples from marine waters of unspecified temperatures. When the M-FC method was further modified to include a 5-h preincubation period at 35°C followed by 18 h at 44.5°C, 90% of the membrane filter counts fell within the 95% confidence limits of corresponding MPN values (9). Numerous seawater samples were analyzed, but the effect of water temperature was not addressed. In contrast to the present

findings, the VRBA overlay technique was observed by others (10) to be more efficient than the MPN for enumerating injured cells from marine environments. However, it is not possible to directly compare results since these workers emphasized recovery of fecal coliforms from frozen and fresh seafood with analysis of a small number of environmental water samples of unspecified temperature. The observed effects of cold estuarine waters on *E. coli* enumeration were similarly observed in freshwater (6). These workers suggested that heat shock was a major factor in decreased membrane filter counts at 44.5°C. Meynell (18) suggested that injury resulting from cold temperature extremes was due to interference with permeability control mechanisms and was reversible. The dramatic loss of adenylates observed after exposure to cold estuarine water is consistent with the finding that chilled suspensions of *Aerobacter aerogenes* suffer leakage of endogenous pools of amino acids and nucleotides (26). The effects of temperature over a complete seasonal cycle on *E. coli* viability in estuarine water are presented elsewhere (3).

Our results suggest that fecal isolates of *E. coli* exposed in situ to estuarine waters at 27°C retain a remarkable ability to regulate energy charge while demonstrating a slow decline in ATP and total adenylate concentrations. In almost all cases, energy charge values were greater than 0.8. Soracco et al. (R. J. Soracco, D. L. Tison, and D. H. Pope, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, N43, p. 170) noted similar results for cells exposed to a thermal effluent in a freshwater pond. Although ATP, total adenylates, and viable count declined, energy charge remained relatively stable.

ATP has been used by many workers as a measure of microbial biomass. This implies the

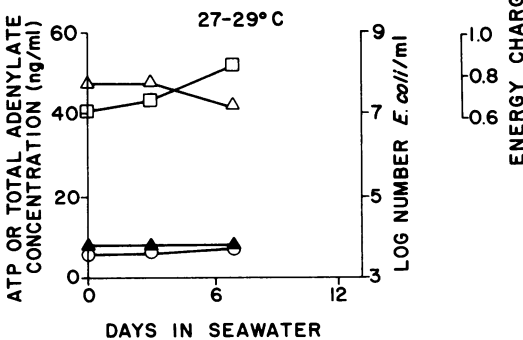


FIG. 6. Incidence of stress as measured by energy charge, ATP, and total adenylates for *E. coli* pregrown in TSB and exposed to 27°C, 20-ppt-salinity seawater. Symbols: □, energy charge; ○, ATP; ▲, total adenylates; △, log number of *E. coli* per ml.

ratio of ATP to cell carbon remains constant (11), regardless of physiological status or environmental stress. It has been suggested that ATP be used to estimate biomass of stressed coliform bacteria in the environment (7, 13). Investigators (7) exposed dialysis tubing containing a pure coliform culture to seawater at 8 to 12°C and observed only a threefold decrease in ATP per cell over 6 days. It was noted that contamination by indigenous microbiota did occur. We believe that the contribution of autochthonous microorganisms such as algae, bacterial predators, and indigenous bacteria to the total ATP measurements may have been underestimated due to the enumeration methodology employed. Our observations and those of Soracco et al. (Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, N43, p. 170) suggest that ATP may not be an accurate measure of biomass for *E. coli* stressed by exposure to aquatic environments.

We have presented evidence that fecal isolates of *E. coli* can be sublethally injured after exposure to a local estuarine environment as evidence by selective versus nonselective enumeration, an electrochemical detection method, and measurement of intracellular nucleotides. Although many factors such as salinity, pollutants, light, algal metabolites, and nutrient deficiencies may contribute to fecal coliform debility, the development and degree of injury observed in our experiments was related to duration of exposure and water temperature. The importance of these findings is that evaluation of methods for enumeration of stressed enteric bacteria must include consideration of seasonal changes in the local physicochemical conditions to which the organisms will be exposed.

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LITERATURE CITED

- American Public Health Association. 1970. Recommended procedures for the examination of seawater and shellfish, 4th ed. American Public Health Association, Inc., Washington, D.C.
- Anderson, I. C., M. Rhodes, and H. Kator. 1979. Sublethal stress in *Escherichia coli*: a function of salinity. *Appl. Environ. Microbiol.* **38**:1147-1152.
- Anderson, I. C., M. Rhodes, and H. Kator. 1983. Seasonal variation in survival of *Escherichia coli* exposed in situ in membrane diffusion chambers containing filtered and non-filtered estuarine water. *Appl. Environ. Microbiol.* **45**:1877-1883.
- Atkinson, D. E. 1969. Regulation of enzyme function. *Annu. Rev. Microbiol.* **23**:47-68.
- Bissonnette, G. K., J. J. Jezeski, G. A. McFeters, and D. G. Stuart. 1975. Influence of environmental stress on enumeration of indicator bacteria from natural waters. *Appl. Microbiol.* **29**:186-194.
- Davenport, C. V., E. B. Sparrow, and R. C. Gordon. 1976. Fecal indicator bacteria persistence under natural conditions in an ice-covered river. *Appl. Environ. Microbiol.* **32**:527-536.
- Dawe, L. L., and W. R. Penrose. 1978. "Bactericidal" property of seawater: death or debilitation? *Appl. Environ. Microbiol.* **35**:829-833.
- Dufour, A. P., E. R. Strickland, and V. J. Cabelli. 1981. Membrane filter method for enumerating *Escherichia coli*. *Appl. Environ. Microbiol.* **41**:1152-1158.
- Green, B. L., W. Litsky, and K. J. Sladek. 1980. Evaluation of membrane filter methods for enumeration of fecal coliforms from marine waters. *Mar. Environ. Res.* **3**:267-276.
- Hackney, C. R., B. Ray, and M. L. Speck. 1979. Repair detection procedure for enumeration of fecal coliforms and enterococci from seafoods and marine environments. *Appl. Environ. Microbiol.* **37**:947-953.
- Holm-Hansen, O. 1973. Determination of total microbial biomass by measurement of adenosine triphosphate, p. 73-89. *In* L. H. Stevenson and R. R. Colwell (ed.), *Estuarine microbial ecology*. University of South Carolina Press, Columbia, S.C.
- Holm-Hansen, O., M. B. Shattuck, and D. M. Karl. 1977. Effects of luciferin concentration on light emission from luciferase-ATP preparations, p. 81-107. *In* G. A. Borun (ed.), *Second Bi-Annual ATP Methodology Symposium*, SAI Technology Co., San Diego, Calif. H. Paul Publishing Co., Pacific Beach, Calif.
- Karl, D. M. 1980. Cellular nucleotide measurements and applications in microbial ecology. *Microbiol. Rev.* **44**:739-796.
- Karl, D. M., and O. Holm-Hansen. 1978. Methodology and measurement of adenylate energy charge ratios in environmental samples. *Mar. Biol.* **48**:185-197.
- Klein, D. A., and S. Wu. 1974. Stress: a factor to be considered in heterotrophic microorganism enumeration from aquatic environments. *Appl. Microbiol.* **27**:429-431.
- McCarthy, J. A., H. A. Thomas, Jr., and J. E. Delaney. 1958. Evaluation of reliability of coliform density tests. *Am. J. Public Health* **48**:1628-1635.
- McFeters, G. A., and D. G. Stuart. 1972. Survival of coliform bacteria in natural waters: field and laboratory studies with membrane-filter chambers. *Appl. Microbiol.* **24**:805-811.
- Meynell, G. G. 1958. The effect of sudden chilling on *Escherichia coli*. *J. Gen. Microbiol.* **19**:380-389.
- Nakada, D., and B. Magasanik. 1964. The role of inducer and catabolite repressor in the synthesis of β -galactosidase by *Escherichia coli*. *J. Mol. Biol.* **8**:105-127.
- Olson, B. H. 1978. Enhanced accuracy of coliform testing in seawater by a modification of the most-probable-number method. *Appl. Environ. Microbiol.* **36**:438-444.
- Presnell, M. W. 1974. Evaluation of membrane filter methods for enumerating coliforms and fecal coliforms in estuarine waters, p. 127-131. *In* D. S. Wilt (ed.), *Proceedings 8th National Shellfish Workshop*, New Orleans, La. Department of Health, Education and Welfare, Food and Drug Administration, Washington, D.C.
- Ray, B. 1979. Methods to detect stressed microorganisms. *J. Food Protect.* **42**:346-355.
- Rose, R. E., E. E. Geldreich, and W. Litsky. 1975. Improved membrane filter method for fecal coliform analysis. *Appl. Microbiol.* **29**:532-536.
- Sokal, R. R., and F. J. Rohlf. 1981. *Biometry*, 2nd ed. W. H. Freeman and Co., San Francisco.
- Stevens, A. P., R. J. Grasso, and J. E. Delaney. 1974. Measurement of fecal coliform in estuarine water, p. 132-136. *In* D. S. Wilt (ed.), *Proceedings 8th National Shellfish Workshop*, New Orleans, La.
- Strange, R. E., and F. A. Dark. 1962. Effect of chilling on *Aerobacter aerogenes* in aqueous suspension. *J. Gen. Microbiol.* **29**:719-730.
- Stuart, D. G., G. A. McFeters, and J. E. Schillinger. 1977. Membrane filter technique for the quantification of stressed fecal coliforms in the aquatic environment. *Appl. Environ. Microbiol.* **34**:42-46.
- Vasconcelos, G. J., and R. G. Swartz. 1976. Survival of bacteria in seawater using a diffusion chamber apparatus in situ. *Appl. Environ. Microbiol.* **31**:913-920.