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ESTUARINE MICROBIAL ECOLOGY

Edited by L. HAROLD STEVENSON *and* R. R. COLWELL



Published for the Belle W. Baruch Coastal Research Institute by the
UNIVERSITY OF SOUTH CAROLINA PRESS
COLUMBIA, SOUTH CAROLINA

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FIRST EDITION

Published in Columbia, South Carolina, by the
University of South Carolina Press, 1973

Manufactured in the United States of America by Kingsport Press, Inc.

Library of Congress Cataloging in Publication Data

Belle W. Baruch Symposium in Marine Sciences, 1st,
University of South Carolina, 1971.
Estuarine microbial ecology.

(The Belle W. Baruch library in marine science, no. 1)

Includes bibliographies.

I. Estuarine ecology—Congresses. 2. Microbial
ecology—Congresses. I. Stevenson, L. Harold, 1940—
ed. II. Colwell, Rita R., 1934— ed. III. Belle
W. Baruch Coastal Research Institute. IV. Title.
V. Series.

QH541.5.E8B4 1971 576'.15 73-9585

ISBN 0-87249-281-8

Salinity and Temperature Interactions and Their Relationship to the Microbiology of the Estuarine Environment¹

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Estuarine Microbial Ecology.

So. Carolina Press, Columbia, S.C.
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An estuary, unlike the open ocean, is a dynamic system that is continuously undergoing changes in its physical and chemical properties. Marked fluctuations in the levels and kinds of nutrients, temperature, and salinity are normally found in estuarine environments. These changes occur principally as a result of fresh-water intrusions, tidal changes, evaporation, seasonal variations, and diurnal insolation. Moreover, imposition by man of thermal, fecal, and industrial wastes has also affected these properties.

In terms of the ecology of aquatic environments, bacteria serve a number of functions, including the mineralization of refractile organic matter, the assimilation of low levels of dissolved organics, and possibly the contribution of essential growth factors to organisms of other trophic levels. In the estuarine environment, it would be expected that all of these microbial activities would be significantly affected by changes in temperature and salinity.

There are numerous reports on the affects of either temperature or salinity on bacteria. However, a temperature response or a salt requirement by a marine bacterium does not validate inferences from the laboratory to the marine environment. Rather than make suppositions about *in situ* microbial activities based on laboratory data, we have been investigating the interrelationship of temperature and salinity on the growth and rates of assimilation of organics by marine psychrophilic bacteria. This report represents results of some of these investigations.

The Salinity Effects on Maximal Growth Temperature

In 1968, Stanley and Morita (6) demonstrated that the maximum growth temperature of *Vibrio marinus* MP-1 was affected by changes in salinity. A similar response was exhibited by three other bacteria isolated from marine sources (Fig. 1). With two strains of *V. marinus*, a difference of about 10 C was found between the maximal growth temperature at the highest and lowest salinities at which growth occurred. The difference was less marked with the two bacterial isolates from the Antarctic. The maximal growth temperature for MP-1 at a salinity of approximately 7‰ was 10 C. However, at a salinity of 35‰ the maximal growth temperature increased to 21.2 C. In all salt-requiring bacteria

¹ Published as special report No. 339, Oregon Agricultural Experiment Station.

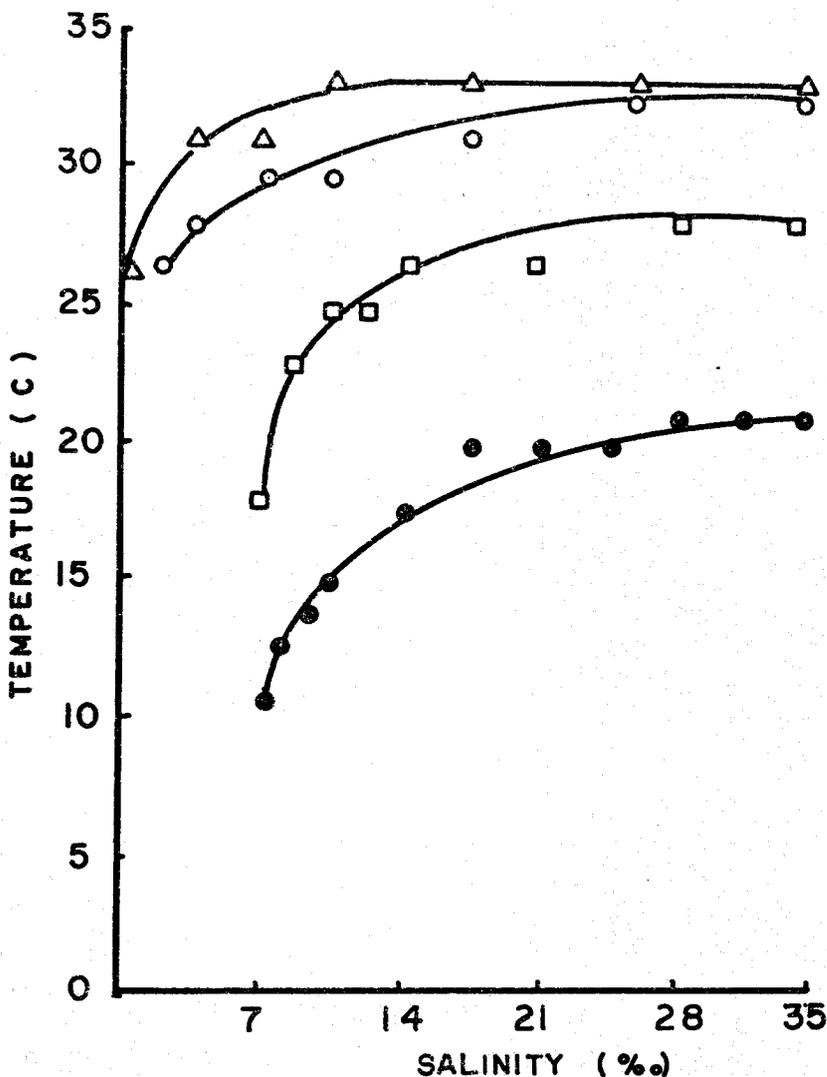


Fig. 1. Effect of salinity of the growth medium on the maximal growth temperature of certain bacteria isolated from the marine environment. In addition to artificial seawater, the medium contained 2.5 g of polypeptone, and 10 ml of vitamin solution dissolved in 1 l of distilled water. The pH was adjusted to 7.4. Symbols: ●, *Vibrio marinus* MP-1; □, *V. marinus* PS 207; ○, 20-PFD/2; △, D30-10WBD/3. (Reprinted from Stanley and Morita (6) by permission of the American Society for Microbiology.)

that have been tested in this laboratory (including *Halobacterium cutirubrum*), we have found that salinity influences the maximal growth temperature.

Further studies were undertaken to determine whether these results were due to an osmotic phenomenon or due to requirements for specific salts. It can be seen from Table 1 that when sodium and lithium chlorides were added to a basal medium containing known quantities of essential salts, growth occurred at 20 C. However, with magnesium chloride the maximal growth temperature was de-

pressed to 16.3 C. No growth was obtained when potassium, rubidium, or ammonium chloride was present. However, in a completely defined medium, lithium chloride could not completely replace the sodium chloride requirement.

Table 1. Effect of different cations on the growth temperature of *Vibrio marinus* (MP-1^a)

Cation Added as the Cl Ion	Maximal Growth Temp (C)
None	No growth
Na ⁺	20.0
Li ⁺	20.0
K ⁺	No growth
Rb ⁺	No growth
NH ₄ ⁺	No growth
Mg ⁺⁺	16.3

^aThe salts were added to the basal medium to give a cation concentration of 0.40 M. Basal medium contained, per liter of distilled water: polypeptone, 2.5 g; NaCl, 0.1 g; K₂HPO₄, 0.1 g; MgSO₄ 7H₂O, 0.1 g; trace element solution, 10 ml; vitamin solution, 10 ml. The pH was adjusted to 7.4. (Reprinted from Stanley and Morita (6) by permission of the American Society for Microbiology.)

In an effort to clarify further the effectiveness of NaCl in raising the maximal growth temperature, MP-1 was grown in different concentrations of sodium chloride in a chemically defined medium (Fig. 2). The lowest concentration of sodium chloride at which growth took place was 0.15 M. At this concentration, the maximum growth temperature was 11.5 C. The maximal growth temperature thereafter increased with increasing concentrations of sodium chloride to reach a maximum of 20.0 C between 0.35 and 0.60 M. Above this concentration, there was a reduction of the maximum growth temperature.

Salinity Effects on Catabolism of Glucose

The effects of salinity on the maximal growth temperature were examined by Griffiths and Morita (2) in terms of nutrient uptake and catabolism. In observing the effects of cations and anions on the uptake of uniformly labeled glucose, they found that the cations that did not permit growth did not permit active uptake of glucose. It was concluded that it was quite possible that previous researchers (6) had actually been looking at a specific cation requirement for nutrient uptake.

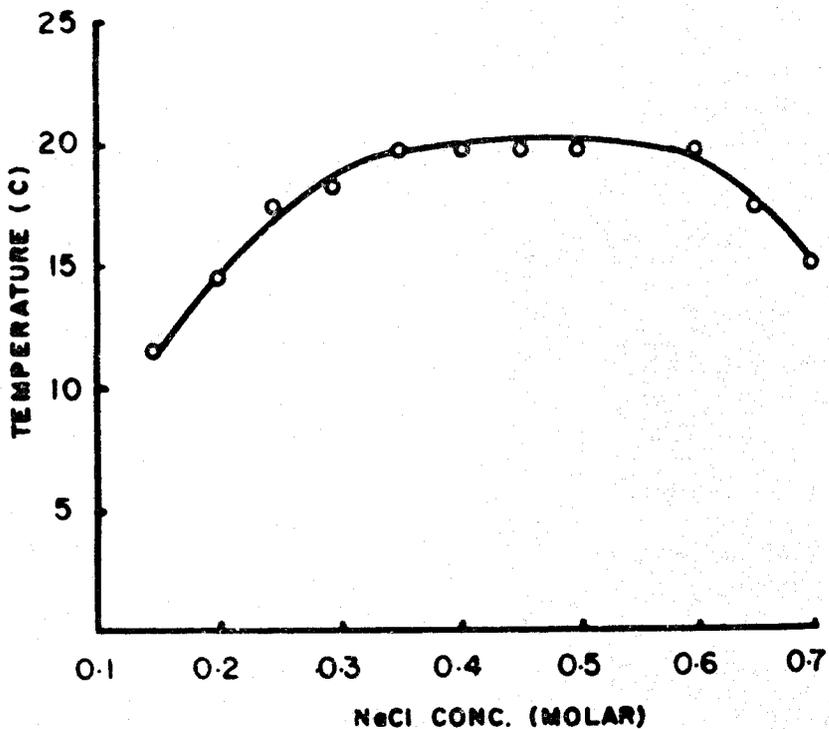


Fig. 2. Effect of NaCl concentration on the maximal growth temperature of *Vibrio marinus* MP-1 in a defined medium. The growth medium was the same as the stock culture medium except that polypeptone was replaced by glucose (2.5 g/l) and KNO_3 (1.0 g/l). (Reprinted from Stanley and Morita (6) by permission of the American Society for Microbiology.)

Griffiths and Morita (2) also looked at the effects of varying salinity on the uptake of glucose. As the salinity was increased from 0.15 M NaCl to 0.30 M NaCl, there was a rapid increase in the amount of glucose taken up. In this salinity range, the change in the observed disintegrations per minute (DPM) per 0.1 M NaCl was 4.33×10^3 . As the sodium chloride concentration was increased from 0.3 to 0.9 M, there was a steady decrease in the observed DPM but this was in the order of $\Delta 0.67 \times 10^3 \text{ DPM} / \Delta 0.1 \text{ M NaCl}$. The first narrow region of rapid change includes the minimum growth salinity (at 15°C). This same rapid shift in uptake was not seen, however, in salinity range of maximum growth, i.e., 0.7 M NaCl. The rapid shift in uptake patterns seen in the salinity range for minimum growth suggests that shifts in respiratory patterns were due to changes in salinity.

Specifically labeled glucose and radiorespirometry were used to follow the effects of salinity on respiration. Of the various labels used, those labeled on the first and sixth carbons of glucose gave some of the most interesting results. As the salinity was increased from 0.15 M to 0.30 M NaCl, there was a rapid decrease in the amount of $^{14}\text{CO}_2$ evolved. A gradual change in cell respiration was seen as the salinity was increased from 0.30 M to 0.90 M NaCl. Even more important was the fact that the relative amount of $^{14}\text{CO}_2$ evolving from the sixth

carbon increased at a faster rate (with increasing salinity) than that from the first carbon. As a result, the $C_6:C_1$ ratios changed from 1.0 at 0.25 M NaCl to 0.19 at 0.90 M NaCl. It may be of significance that the $C_6:C_1$ ratio is one at a point just below the minimum salinity for growth. Thus, it was postulated that salinity was altering the relative participation of the pentose pathway resulting in a shift in the respiratory pattern.

It was assumed that all of the glucose was catabolized via the Embden-Meyerhof and pentose pathways. They argued that if the amount of CO_2 evolved from the third and fourth carbons of glucose was approximately equal and much greater than that evolved from the first and sixth carbons, then the Embden-Meyerhof pathway was being utilized for glucose catabolism. However, any significant difference in the levels of CO_2 evolved from the first and sixth carbons reflects the relative participation of the pentose pathway in the catabolism of glucose. If these assumptions are true, it would appear that the salt concentration affected the amount of glucose catabolized through the pentose pathway.

Salinity-Temperature Effects on Protein Synthesis

In an effort to elucidate further the effects of salinity-temperature interaction on the physiology of named microorganism, Cooper and Morita (1) examined the effects of salinity-temperature interactions on the net protein synthesis, RNA synthesis, and viability of *V. marinus*.

Cultures of *V. marinus* were grown to mid-log phase, harvested, washed by filtration, and resuspended in fresh medium. Growth curves of this organism were shown to be nearly identical at salinities of 25 through 35‰. However, growth was negligible at lower salinities. Proline and uracil, radioactive precursors to protein and RNA respectively, were added to the medium at temperatures from 15 to 25 C. The cell suspensions were assayed for protein or RNA synthesis. At a salinity of 25‰, protein synthesis occurred at 15 and 20 C but was significantly depressed at 25 C. Similar results were obtained at salinities of 30 through 40‰.

Protein synthesis and precursor uptake by whole cells was determined at one-degree temperature intervals between 20 and 25 C at various salinities. These results are shown in Figure 3. At a salinity of 25‰, protein synthesis was similar at 20 and 21 C, but a marked decrease in protein synthesis occurred at 22 C. Precursor uptake into whole cells continued after protein synthesis had decreased. However, this marked decrease in protein synthesis at 22 C was not observed at a salinity of 30‰. Instead there occurred a gradual decrease in protein synthesized with increasing incubation temperature.

At a salinity of 35‰, no decrease in protein synthesis was seen until after 20 min incubation at 24 C. Again precursor uptake continued after protein synthesis had decreased. It can be concluded that the cell was taking up proline, but the proline was not being incorporated into the protein within the cell.

At a salinity of 40‰, however, precursor uptake decreased before protein synthesis decreased at the incubation temperatures of 24 and 25 C. Here a different effect was probably preventing the incorporation of labeled proline into protein.

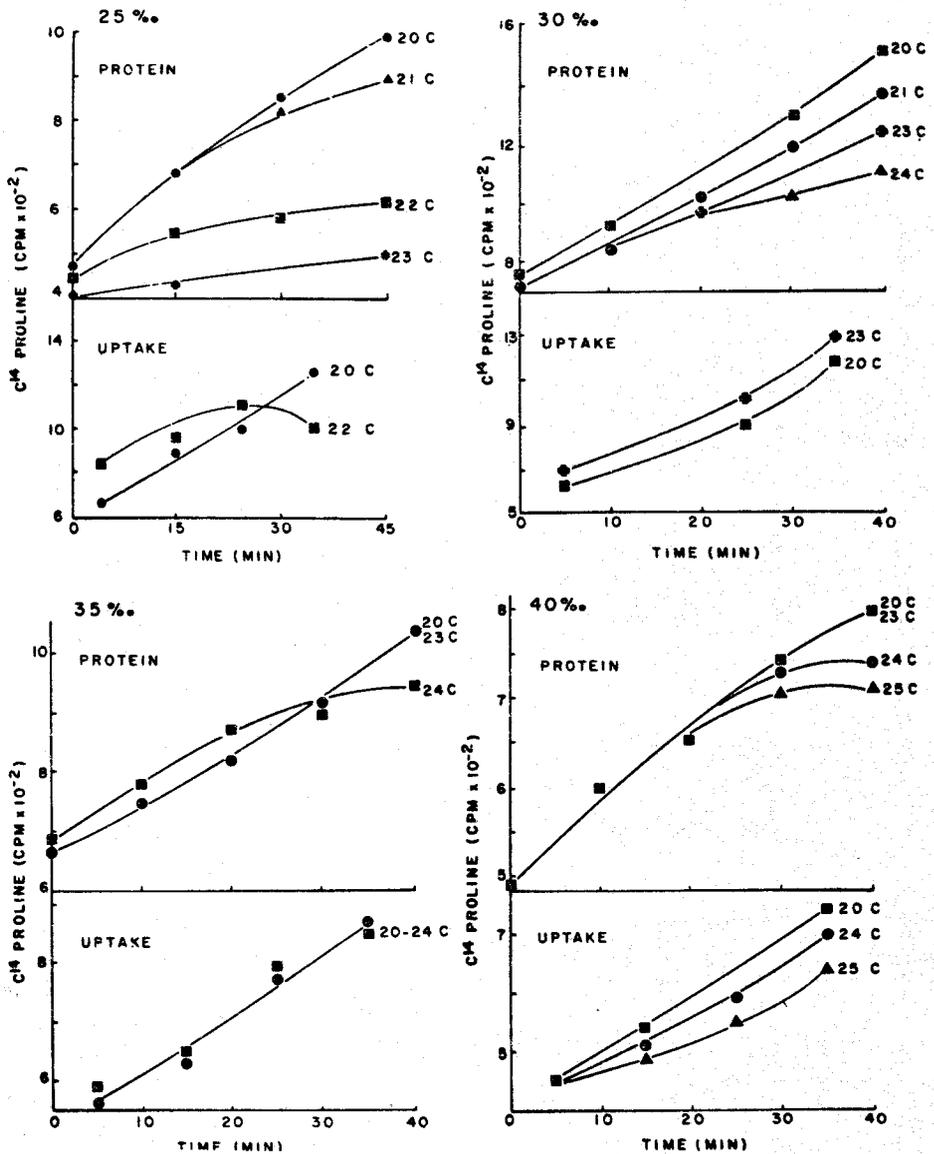


Fig. 3. Incorporation into protein (top of each graph) and whole-cell uptake (bottom of each graph) of ^{14}C -proline by *V. marinus* MP-1 incubated in SSPU medium at various salinities. Volume of cell suspension was 1.0 ml. (Reprinted from Cooper and Morita (1) by permission of the American Society of Limnology and Oceanography, Inc.)

Thus, the data would indicate that at salinities of 25 through 35‰, precursor entered the cells and was available for protein synthesis. However, thermal affects probably were preventing the uptake of label into the cells at a salinity of 40‰.

To determine if a thermal lesion occurred in translation or in transcription, concurrent RNA and protein synthesis studies were performed at salinities of 15 to 40‰. In Figure 4, it is clearly shown that at a salinity of 20‰, the amount

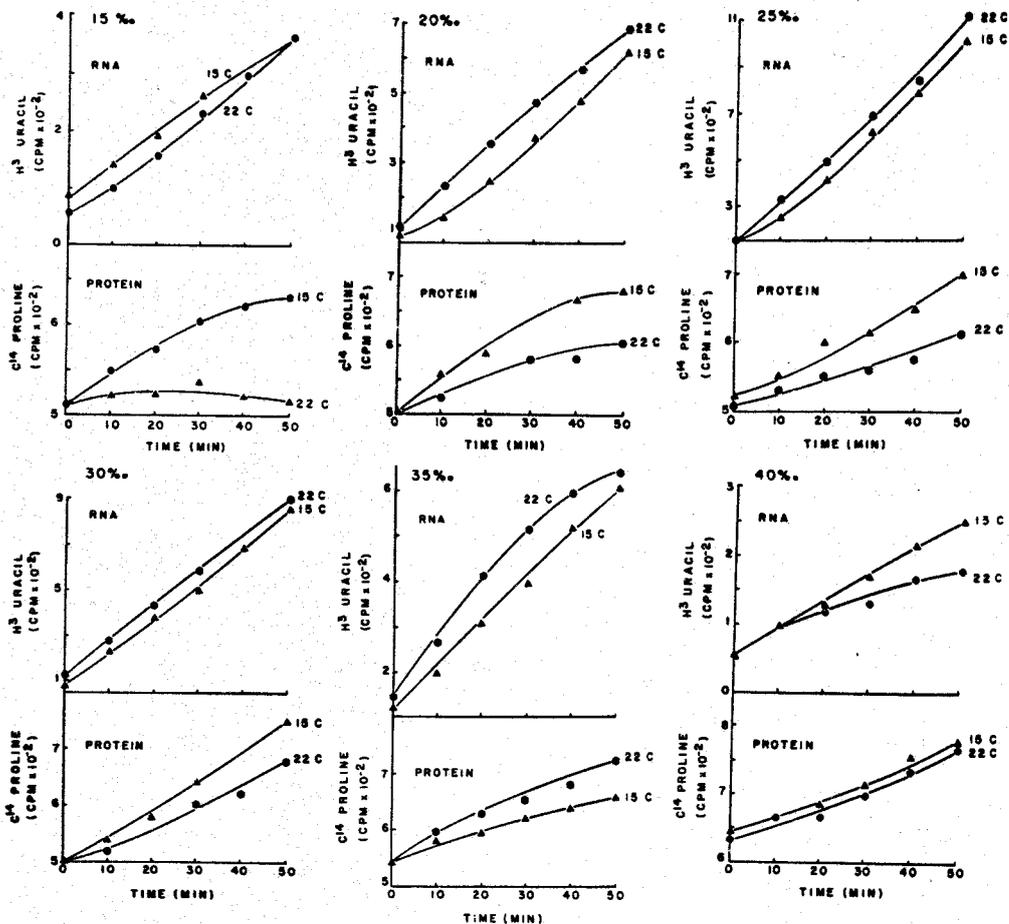


Fig. 4. Incorporation of ^3H -uracil into RNA (top of each graph) and ^{14}C -proline into protein (bottom of each graph) during incubation of *V. marinus* MP-1 in SSSU medium at various salinities. Volume of cell suspension was 0.5 ml. (Reprinted from Cooper and Morita (1) by permission of the American Society of Limnology and Oceanography, Inc.)

of RNA synthesized during the first 40 min was greater at 22 than at 15 C. At this salinity, protein synthesis was less at 22 than at 15 C. Identical results were observed with salinities of 25 and 30‰.

At a salinity of 35‰, both protein and RNA synthesis were greater at 22 than at 15 C, whereas at a lower salinity, protein synthesis was less at 22 than at 15 C. These studies indicate that the thermal lesion occurred at the translation level of protein synthesis.

At salinities of 15 and 40‰, however, RNA synthesis was less at 22 than at 15 C. This would indicate that extremes of temperature and salinity also decrease the total amount of label incorporated into RNA.

Temperature-Salinity on Glutamic Dehydrogenase Synthesis

Staley and Morita (5) have shown that temperature and salinity can influence the synthesis of glutamic dehydrogenase (GDH) in *V. marinus*. The scheme in-

volving GDH synthesis was selected because of the strategic position of the enzymes in the TCA cycle.

Cells used for the experiments were first grown at 15 C for 48 hr in a defined glucose-ammonium medium (GAM). They were harvested, washed in a phosphate-salts buffer, and resuspended. Aliquots of the washed cell suspension were added to flasks containing glutamate medium (GM) at a variety of salinities and were equilibrated to given temperatures. After shifting the glucose-grown cells to the GM, portions were removed at appropriate time intervals. The cells were lysed by sonic treatment and the cell-free extracts assayed for GDH activity. Cells not added to GM were taken as the control samples (i.e., no exposure to glutamate). In this way Staley and Morita (4) were able to measure the effects of temperature and salinity on the synthesis of the enzyme over a period of time. Other studies by Staley (4) indicate that the synthesis of GDH is also affected by temperature so that a temperature-salinity interaction comes into play.

As indicated in Figure 5, the rate of GDH synthesis in cells grown for 48 hr at 15 C and shifted to GM at 15 C at various salinities was apparently greatest in 0.4 M NaCl. When cells were exposed to sodium chloride concentrations below the optimal for GDH synthesis (i.e., 0.26 M), the cells showed a reduced ability to synthesize GDH. Since the substrate uptake rate, (Fig. 6) as well as the rate of protein synthesis (Fig. 7), was maximal at this salinity, the organisms were quite capable of getting the substrate into the cells as well as synthesizing GDH. Net synthesis occurred at a very low rate. A correlation of Figure 5 with Figures 6 and 7 cannot be made. This probably indicates that the salinity effect on the synthesis of this protein (GDH) is not related to the salinity effect on uptake of glutamic acid or the synthesis of net protein by the cell. In other words, precisely how salinity affects the synthesis of GDH remains to be established.

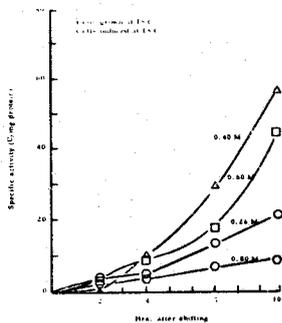


Fig. 5. Synthesis of glutamic dehydrogenase in *V. marinus* when cells were shifted from GAM to GM medium containing various molarities of NaCl at 15 C.

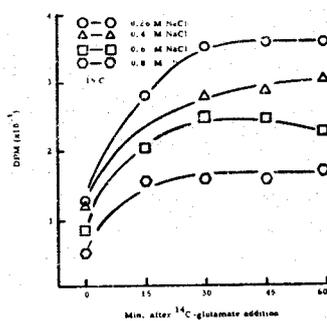


Fig. 6. Time course of ^{14}C -glutamate ($0.2 \mu\text{c/ml}$ cell suspension) uptake by *V. marinus* cells in GM medium (3.0 mM glutamate) at 15 C at various NaCl concentrations.

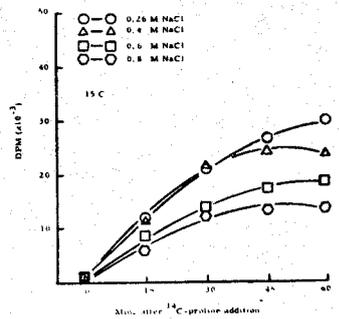


Fig. 7. Time course of ^{14}C -proline ($0.05 \mu\text{c/ml}$ cell suspension) incorporated by *V. marinus* in GM medium (3.0 mM glutamate) at 15 C at various NaCl concentrations.

Concluding Remarks

We have shown that salinity plays an important role in governing the temperature range of growth of certain bacteria and we have tried to elucidate the mechanisms responsible for the effect observed in temperature-salinity interactions. From a more practical point of view, it is of interest to speculate on the physiological reaction of a marine bacterium subjected to salinity and temperature variations and to the nutritionally richer waters of the estuary. Weimer and Morita (7), using an inducible enzyme system, showed with *Vibrio* MP-41 that the activity of the exoenzyme, gelatinase, was adversely affected by varying salt concentrations up to 15%.

Greatest activity was observed in the absence of salts and this activity decreased with increasing salinity. Increasing the salt concentration above 3.6%, however, caused little further decrease in activity. Thus, increasing the salinity in the presence of cooler water could put a limit on the potentialities of this exoenzyme.

Conversely, what would happen to a marine bacterium subjected to an influx of relatively warm, fresh water? In Figure 8, Haight and Morita (3) show that there is leakage of protein, RNA, DNA, and amino acids into the surrounding medium when an organism (in this case, *V. marinus* MP-1) is subjected to temperatures above 20 C. Such events could occur when organisms present in a salt wedge are forced to the surface in an estuary. The data in Figure 9 demonstrate how organisms can be subjected to changes in salinity and temperature resulting in the lysis of certain marine microorganisms such as the psychrophile *V. marinus*

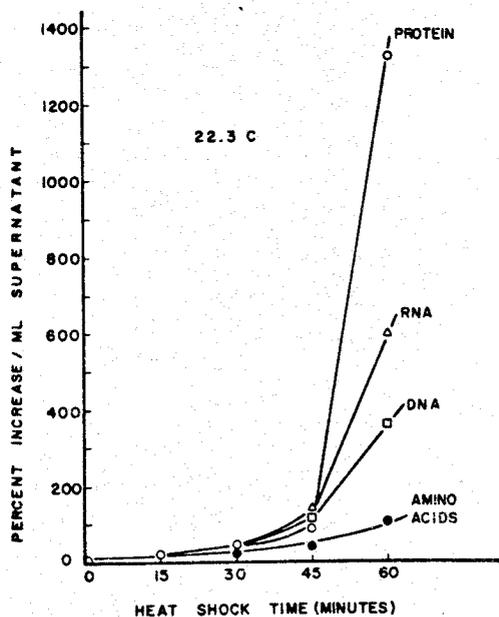


Fig. 8. Heat-induced leakage from *Vibrio marinus* MP-1 at 22.3 C. (Reprinted from Haight and Morita (3) by permission of the American Society for Microbiology.)

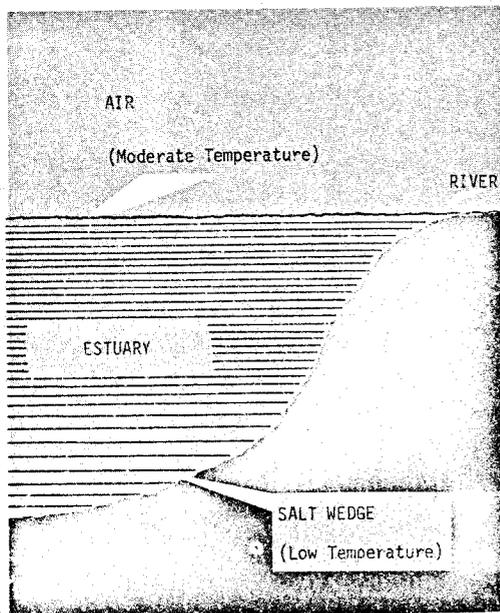


Fig. 9. Diagrammatic sketch of a salt wedge surfacing in an estuary.

MP-1. Lowering salinity also enhances leakage of intracellular material from the cell. However, this phenomenon is not completely detrimental to the ecology of the estuary. In fact, it probably adds to the overall productivity by releasing vitamins, enzymes, purines, and pyrimidines—materials known to affect growth in other organisms.

For these and other reasons, tides are important in the recycling process and in the general ecology of estuaries. The movement of tides not only affects the temperature and salinity of the environment but also the amount and types of nutrient compounds found in the water. Tides contribute large amounts of nutrients to the offshore environments, thus allowing them to enter the food cycles essential to productivity in all waters.

Morita and Kalber* took samples of water at 1-hr intervals during tide changes in a mud flat near Freeport, Texas, on two occasions. The overall regeneration or utilization of phosphate and ammonia was measured by the difference between the initial figure and that present in the water after a 15-min incubation period. The data clearly demonstrate that the regeneration or utilization of ammonia and phosphate in this environment varied from hour to hour and that more ammonia and phosphate were found in bottom water than in surface water (Fig. 10). These data also indicate that within an intertidal area, the water is heterogenous in terms of ammonia and phosphate content and also in utilization and regeneration of ammonia and phosphate. The mud flat thus represents a dynamic situation where the ammonia and phosphate content of the water is constantly undergoing changes.

It is important to note, moreover, that although there have been many studies enumerating microorganisms in a given estuarine environment, numbers alone do not tell us the rate of *in situ* activity taking place. The presence of any physiological group of bacteria only indicates that the potential for microbial activity exists. This activity may not be expressed, however, until the conditions are suitable. It is important to understand that conditions of flux exist at all times in a complex environment such as an estuary.

With this in mind, we believe that the Wright-Hobbie kinetic approach, discussed by Dr. Wright in this symposium, is very useful in helping to understand the total environment and the activities taking place therein. Our work on Upper Klamath Lake in Oregon** and in the Antarctic† illustrates some applications of a kinetic approach. This approach takes into consideration the indigenous microflora in relation to the other environmental conditions such as temperature, salinity, and primary nutrients.

We thus conclude that marine microorganisms are always vulnerable to salinity and temperature changes that may significantly affect their normal functions and consequently affect the environment. A knowledge of the results of these changes on metabolic potentiality (i.e., formation of adaptive enzymes and exoenzymes, rate of metabolism, etc.) of marine organisms under near-shore environmental conditions is of paramount importance if we are to understand and more wisely participate in the dynamic economy of the oceans.

* Unpublished data.

** Burnison, Gillespie, Harrison, Wright, and Morita, unpublished data.

† Gillespie, Jones, and Morita, unpublished data.

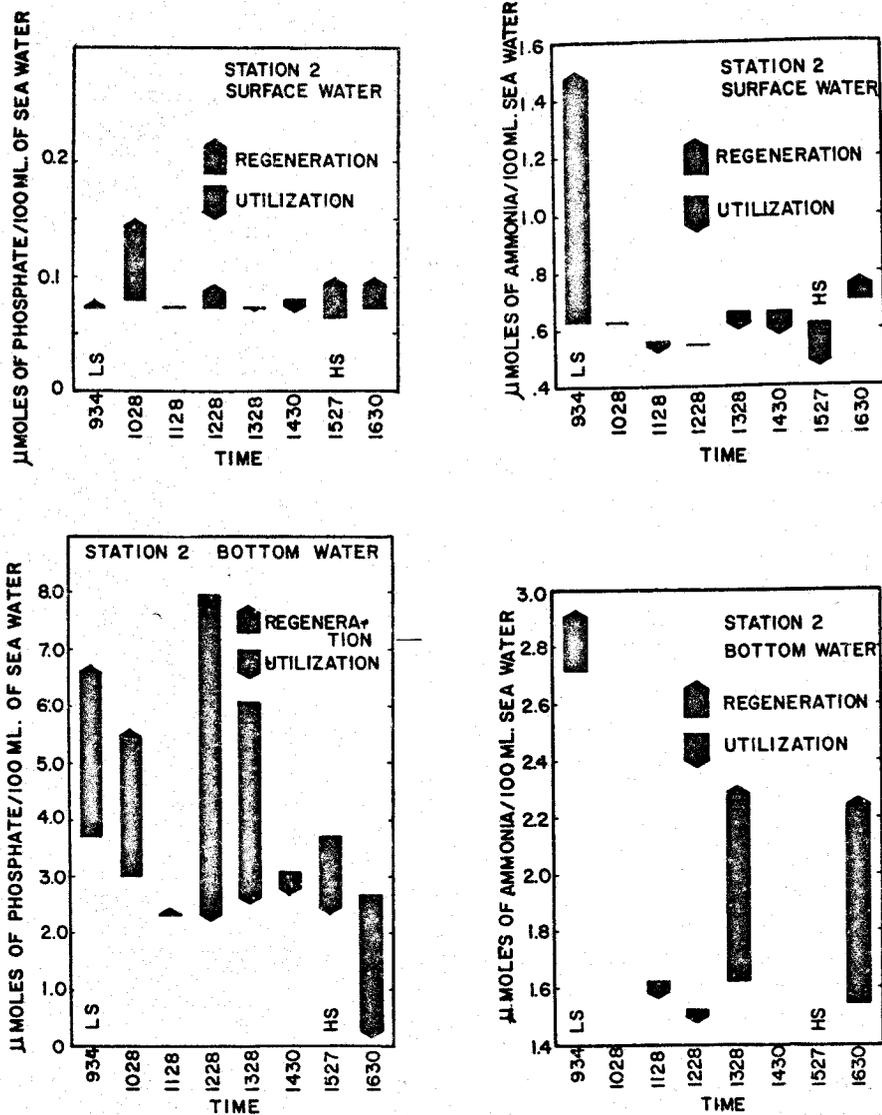


Fig. 10. Regeneration and utilization of phosphate and ammonia in various samples of seawater at Station II (mud flat near Freeport, Texas) during a tidal cycle. Water samples were taken and the analyses made at times indicated (0-time). Analyses were also made on subsamples of water after incubation for 15 min. The difference in phosphate and ammonia levels between analyses after 15-min incubation and 0-time gives the amount of regeneration (+ value) and utilization (- value). Incubation was in the dark at approximately the same temperature of the water. HS = high slack water; LS = low slack water.

Acknowledgment

The research reported in this paper was supported in part by the Oceanography Section, NSF, NSF grant GA-28521 and in part by PHS Training Grant 5 T1 GM 704-08 MIC from the National Institute of General Medical Sciences.

The senior author wishes to express his sincere thanks to his former and current students and associates for some of the data presented in this paper.

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