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NUTRITIONAL STUDIES OF MARINE FUNGI IMPERFECTI WITH EMPHASIS ON NITROGEN NUTRITION IN *ZALERION MARITIMUM*

by

Leslie Marian Churchland

B.A., University of British Columbia, 1966
M.Sc., Simon Fraser University, 1971

A THESIS SUBMITTED IN PARTIAL FULFILLMENT

OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in the Department

of

Biological Sciences

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Simon Fraser University

September, 1976

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Nutritional Studies of Marine Fungi Imperfecti with Emphasis on Nitrogen Nutrition in Zalerion Maritimum

ABSTRACT

A continuous culture system was designed for the cultivation of marine fungi. Growth of *Humicola alopallonella* Meyers and Moore, *Monodictys pelagica* (Johnson) Jones, and *Zalerion maritimum* (Linder) Anastasiou was higher in continuous culture than in batch culture. Experiments were carried out to explain the poorer growth in batch culture. Experiments with reinoculated media showed that non-volatile staling products were not present. Nutrient exhaustion was a factor in *Humicola alopallonella*. In all species, low pH conditions in ammonium containing media limited growth. Improved pH control was obtained by substituting MES (2-[N-morpholino] ethanesulfonic acid) for TRIS (tris [hydroxymethyl] aminomethane). Chemical analyses of nutrients in the nutrient solution indicated that phosphate was depleted in batch culture.

Two isolates of *Z. maritimum*, FMC and H 135, were used to study nitrogen nutrition. Both isolates grew on each of NH_4Cl, $NaNO_3$, and $NaNO_2$. Of 21 amino acids tested, L-alanine and L-arginine supported best growth of the FMC isolate. L-valine, L-alanine, L-glutamic acid, L-aspartic acid, L-asparagine, and L-arginine supported good growth of H 135. Both isolates grew on peptone, urea,

citrulline, and allantoin. Results from the continuous system differed both qualitatively and quantitatively from batch culture results. In the FMC isolate, there was greater growth in batch culture on L-glutamic acid than on the three inorganic nitrogen sources. In continuous culture, growth was equal on all nitrogen sources.

Nitrogen compounds were supplied in combination, and their removal from the nutrient solution measured chemically. Both isolates took up ammonium in preference to nitrate. Although both isolates took up ammonium and nitrite, ammonium was removed at a faster rate. Isolate FMC preferentially took up L-glutamic acid when supplied with nitrate. Isolate H 135 took up both compounds, the former at a faster rate than the latter. Both isolates took up ammonium and a group of eight amino acids, when supplied in combination.

Neither isolate demonstrated nitrogen fixation, denitrification, or nitrification. *Zalerion maritimum* had a pattern of nitrogen nutrition similar to that of the terrestrial fungi.

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GENERAL INTRODUCTION

The study of lignicolous marine fungi was begun by Barghoorn (1944) and Linder (1944) in a combined study of the taxonomy and biology of marine fungi. This work included extensive taxonomic and physiological information, and was followed by the "descriptive phase" of marine mycology. In the next twenty years investigators described and listed species from various habitats and geographical locations. Taxonomic and floristic studies are still an important part of marine mycology, because all the possible substrates for marine fungi have not yet been investigated.

During this period, mycologists also investigated the effects of certain marine variables on the growth and distribution of species. Temperature, salinity, and the combined effects thereof were studied, both in the laboratory (Ritchie, 1957; Ritchie and Jacobsohn, 1963) and in the field (Gold, 1959). Substrate preferences observed in the field were tested in the laboratory, and several species were shown to degrade cellulose (Meyers and Reynolds, 1959; Meyers, Prindle, and Reynolds, 1960; Moore and Meyers, 1962).

Hughes (1975) has written a comprehensive review of studies since 1961 on lignicolous, caulicolous, and foliicolous species. In recent years, the emphasis has shifted from descriptive to nutritional and physiological studies. The growth of marine fungi on various carbon

and nitrogen sources (Alderman and Jones, 1971; Sguros, Rodrigues, and Simms, 1973) and the ionic constituents of seawater (Sguros and Simms, 1964; Jones and Jennings, 1965) have been investigated. The salinity requirements of both higher (Tubaki, 1966; Jones, Byrne, and Alderman, 1971; Davidson, 1974a; Schaumann, 1974) and lower (Alderman and Jones, 1971; Harrison and Jones, 1974; Bremer, 1974) marine fungi have been studied. Traditionally, either radial growth or dry weight measurements have been used to measure the growth of marine fungi. Spore production (Meyers and Hoyo, 1966; Byrne and Jones, 1975b) and spore germination (Byrne and Jones, 1975a) are currently being used as additional measures of physiological response.

In the 1970's, there has been increased interest in marine fungal biochemistry. The production of metabolites such as choline sulfate ester (Catalfomo et al, 1972-1973), ergosterol, and choline (Kirk and Catalfomo, 1970) have been documented. Pathways of carbohydrate metabolism such as the Tricarboxylic Acid Cycle (Vembu and Sguros, 1972) and the Hexose Monophosphate Pathway (Holligan and Jennings, 1972b; Lowe and Jennings, 1975) have been investigated. The marine fungi have been shown to degrade such environmentally important compounds as cyclodiene pesticides (Quevedo, Sguros, and Thomann, 1973).

Despite our increasing knowledge of the biochemistry of marine fungi, their role in certain essential marine processes is unknown.

One of the most important challanges in marine mycology is to create laboratory conditions which approximate the marine environment, and thus allow an appraisal of the role of these organisms in their natural habitat. The goals of this thesis were as follows:

1. To develop a continuous culture system for the cultivation of filamentous marine fungi (Chapter I).

2. To compare growth in continuous and batch culture and to determine what factor or factors affect growth in batch culture (Chapter I).

3. To use the continuous system and the batch system to study nitrogen nutrition in Zalerion maritimum (Chapter II).

4. To propose a hypothetical role for Z. maritimum in the marine nitrogen cycle, taking into account the difficulties of extrapolating from in vitro studies to the marine environment (Chapter II).

CHAPTER 1

Growth of *Zalerion maritimum*, *Humicola alopallonella*, and *Monodictys pelagica* in continuous and batch culture.

INTRODUCTION

In early nutritional studies of marine fungi, growth was measured as increases in colony diameter on agar (Barghoorn, 1944; Johnson, Ferchau, and Gold, 1959). This technique had the disadvantages that the nutrient solution could not be chemically defined during growth, pH changes could not be monitored or adjusted, and no correlation could necessarily be made between radial growth and fungal biomass (Mandels, 1965). Radial growth measurements are not generally used for nutritional studies today, although they have been used to study the effects of temperature and salinity on growth (Tubaki, 1966; Schaumann, 1974). The radial growth technique was replaced by dry weight determination, in which the mycelium was removed from a static liquid culture medium, dried, and weighed (e.g. Gustafsson and Fries [1956]). This technique was then modified by incubating the culture flasks on a shaker. This "batch culture" technique is now generally used for nutritional studies of the marine fungi (Jones and Jennings, 1965; Meyers and Hoyo, 1966; Sguros *et al*, 1973; Davidson, 1974a).

In this study, I attempted to increase the growth rates of marine fungi grown in a chemically defined nutrient solution. I designed a continuous flow system for the cultivation of filamentous marine fungi, and compared the growth therein with growth in batch culture.

Fungal growth may affect the chemical environment in batch culture. First, one or more nutrients may be exhausted. Second, depending upon the nitrogen source pH changes may occur. Growth on ammonium lowers pH, whereas growth on nitrate and organic nitrogen raises pH (Sguros and Simms, 1964). These changes may occur despite the addition of buffer, or the buffer itself may be toxic to the fungus (Cochrane, 1958) in concentrations high enough to compensate for the pH effect. Third, byproducts of fungal metabolism may affect growth in a closed system. Nitrite may accumulate and be toxic in acidic media containing NH_4NO_3. Rapid growth may be followed by the production of staling products, metabolites which inhibit fungal growth. Because marine fungi are naturally bathed in a renewable nutrient solution, the use of a continuous culture system may more accurately determine their nutritional requirements. A continuous system should, theoretically, supply fresh nutrients, control pH changes, control osmotic changes, and prevent the accumulation of toxic metabolites.

Terrestrial fungi such as _Coprinus_ (Fries, 1956), _Aspergillus_ (Ng, Smith, and McIntosh, 1973), and _Fusarium_ (Steensland, 1973) have

been grown in continuous culture. Johnson and Gold (1959) designed a technique for the continuous recycling of seawater over marine fungi growing on wood. However, all previous nutritional studies of marine fungi have been carried out on agar or in batch culture.

Investigation of continuous culture techniques led to the question of growth dynamics; why did growth stop in the closed system? Was it due to nutrient exhaustion; if so, of which nutrient or nutrients? Did staling products inhibit growth? Did these species grow determinately as do higher plants and some other fungi (Gottlieb, 1971)? To answer these questions I compared growth in batch and continuous culture, reinoculated used media with fresh mycelium, and analyzed spent medium for glucose, ammonium, nitrate, nitrite, and phosphate.

The three species used for the nutritional experiments were Monodictys pelagica (Johnson) Jones, Humicola alopallonella Meyers and Moore, and Zalerion maritimum (Linder) Anastasiou. Each has been isolated commonly from cellulosic substrates in B. C. coastal waters (Meyers and Reynolds, 1960; Hughes, 1969; Anastasiou and Churchland, 1969; Churchland and McClaren, 1973). Zalerion maritimum and M. pelagica have been isolated from north and south temperate waters, and occasionally from tropical and subtropical seas (Hughes, 1974). Humicola alopallonella has been as frequently isolated from tropical as temperate waters (Hughes, 1974). All three species are "marine"

sensu Kohlmeyer (1974), in that they both grow and reproduce in the marine environment. *Monodictys pelagica* might be better termed a "saltwater" fungus, as it has been found in a saline lake in Wyoming (Davidson, 1974b).

The majority of the work in this thesis was done with the marine Imperfect species *Zalerion maritimum* (Linder) Anastasiou. Linder (1944) originally described this species as *Helicoma maritimum*, and separated it from *H. salinum* by the number of coils and septations in the spores. In 1962 Moore and Meyers placed these two species in the genus *Zalerion*, which differed from *Helicoma* by the presence of constricted septa in the spores and by the number of gyres in the spores. The genus was then divided by Moore and Meyers into four species (*Z. eistla*, *Z. neppura*, *Z. xylestrix*, and *Z. raptor*), based upon colonial morphology, pigmentation, and cellulose utilization. Subsequently, Anastasiou (1963) recombined the four species into one species, *Zalerion maritimum*, which included the *H. maritimum* and *H. salinum* described by Barghoorn and Linder. Anastasiou considered Meyers' four species of *Zalerion* to be physiological variants, and this interpretation is now generally accepted (Tubaki, 1966; Hughes, 1975). Considering the physiological variability of this species I have used two isolates, differing in colonial morphology and pigmentation, for most of the nutritional studies.

The goals of the experiments described in this chapter were to design a continuous culture system for the growth of marine fungi, to compare growth in continuous and batch culture, and to determine what factor or factors affected growth in batch culture. These experiments were designed to increase our understanding of the growth of marine fungi, and allow the formulation of more balanced culture media for future nutritional studies.

MATERIALS AND METHODS

A. Isolation of test species from the marine environment

Monodictys pelagica H 317

Monodictys pelagica was isolated from a hemlock (*Tsuga heterophylla* [Raf.] Sarg.) panel submerged at 1 m for 2 months at Horseshoe Bay, British Columbia. The panel was collected in August, 1969.

Humicola alopallonella H 571

Humicola alopallonella was isolated from a cedar (*Thuja plicata* Donn.) panel submerged at 1 m for 4 months at Cotton Point, Keats Island, British Columbia. The panel was collected in October, 1969.

Zalerion maritimum H 135

Zalerion maritimum H 135 was isolated from a Douglas Fir (Pseudotsuga menziesii [Mirb.] Franco) panel submerged at 1 m for 1 month at Horseshoe Bay, British Columbia. The panel was collected in July, 1969.

Zalerion maritimum FMC

Zalerion maritimum FMC was isolated from a hemlock panel submerged at 1 m for one month at Lions Bay, British Columbia. The panel was collected in July, 1971.

After removal from the sea, the panels were incubated (Johnson and Sparrow, 1961) 1-3 weeks at 20 C. A hypha was then removed from the panel and placed on seawater agar (Johnson and Sparrow, 1961). After autoclaving, 0.5 g/l of streptomycin sulfate and 0.5 g/l of Penicillin G had been added to the seawater agar. A single hyphal tip was taken from the edge of the resultant colony and placed on seawater agar without antibiotics. The cultures were maintained on seawater-yeast extract slants at 4 C. To demonstrate variation in morphology and pigmentation, the two isolates of Zalerion were photographed after 3 weeks' growth on seawater-yeast extract agar at 20 C.

B. Media

The chemically defined liquid culture medium used in the nutritional studies was that of Sguros and Simms (1963), and contained glucose, 9.0 g; NH_4NO_3, 0.4 g; $K_2HPO_4 \cdot 3H_2O$, 0.06 g; THAM (tris [hydroxymethyl] aminomethane), 1.2 g; vitamins, trace elements, and 1000 ml of artificial seawater (Lyman and Fleming, 1940). The vitamins were added from a stock solution to give concentrations of 20 μg/l of thiamine, biotin, pyridoxine, riboflavin, pantothenic acid, ascorbic acid, inositol, choline, niacin, p-aminobenzoic acid, and vitamin B-12. The trace elements were added from a stock solution to give a concentration of $FeCl_3$, 1400 μg/l; $CuSO_4$, $ZnSO_4$, MoO_3, 10 μg/l; $MnSO_4$, 24 μg/l. The artificial seawater was made using distilled and deionized water. The nutrient solution (hereafter referred to as NSA) was adjusted to pH 7.5 (Radiometer pH meter, model 22) by the addition of 1N HCl, filter sterilized (Millipore GS, pore size 0.22μ), and added aseptically to sterile Erlenmeyer flasks. Fifty ml of NSA were added to each 250 ml flask, except when otherwise stated.

C. Inoculum preparation technique

Inoculum preparation techniques followed that of Sguros, Meyers, and Simms (1962). Inoculum was taken from a seawater yeast extract slant, placed on the center of a plate of F 1003 agar (Sguros <u>et al</u>,

1962), and incubated at 20 C for 3 weeks. A segment 0.5 mm in diameter was then taken from the edge of the colony and placed in 125 ml flasks containing 25 ml of NSA. The flasks were then incubated at 20 C for 43 days. This differed from Sguros et al (1962) in the use of a chemically defined medium for the static culture phase. The inoculum was then removed from the 125 ml flasks, added to 50 ml of artificial seawater (ASW) in a Sorvall "Omnimixer", and macerated. The average dry weight/ml of an inoculum sample was determined. The dry weight of the remaining mycelial suspension was then adjusted to 4 mg/ml by dilution with ASW. One ml of this suspension was added to 50 ml of NSA in 250 ml flasks, and incubated on a shaker rotating at 66 rpm at room temperature, 23 ± 2C. After 7 days growth the mycelium was again removed, added to 50 ml ASW, and macerated for 20 seconds. The mycelial suspension was then centrifuged at 5,000 rpm for 30 minutes, washed, centrifuged again, rewashed, and the dry weight of the suspension adjusted to 4 mg/ml. One ml of this suspension was used to inoculate each flask in the nutritional experiments. Inoculated flasks were incubated at 23 C on a shaker rotating at 66 rpm for 7 and/or 14 days. Dry weight was determined by filtering the mycelium through preweighed glass fibre filters and washing thoroughly with distilled water. Filters were dried for 24 hours in a 60 C oven, and the pH of the filtrate measured.

No more than three consecutive transfers were made on liquid culture medium. This resulted from my observation that pellet

morphology and/or pigmentation in *Z. maritimum* changed after a series of transfers in liquid culture media. Inoculum was stored 4 days or less at 4 C.

Because of the quantitative variations between inoculations of mycelia (*cf.* Davidson, 1974) comparisons in this study were made within experiments only.

Throughout this study, and throughout other nutritional and physiological investigations of the fungi, the term "growth" was frequently used in place of the term "dry weight". Although dry weight is the most widely used and generally applicable method of estimating growth (Mandels, 1965), dry weight is not synonymous with growth. When grown in liquid culture *Z. maritimum*, *H. alopallonella*, and *M. pelagica* formed from one to several pellets which increased in volume. Whereas a dry weight measurement represented the total biomass of the pellet, growth occurred only at the hyphal tips. In older hyphae a dry weight measurement may have partially represented the accumulation of polysaccharides, lipids, or wall materials rather than an increase in living protoplasm (Cochrane, 1958). Conversely, protein synthesis may have continued after dry weight increases had stopped (Cochrane, 1958). The term "growth" when used in this thesis should be translated as "the best estimate of growth", obtainable by measuring the dry weight of the mycelium.

D. Continuous culture apparatus

The continuous culture apparatus (Churchland and McClaren, 1976) was modified from Kubitschek (1970) and Fries (1956). The system is illustrated in Figs. 1-2. The 6L reservoir flask (B) flowed at a constant rate despite changes in the height of the nutrient solution. As nutrient solution entered the culture flask at E, air was drawn into the reservoir bottle through a sterile cotton filter at A. The flow rate was controlled by the height of the pressure head (the distance between the end of the air inlet tube and E) and the length and bore of the capillary tubing (D). A pressure head of 42 cm and capillary tubing of 10 cm length and 0.2 mm bore provided a flow rate of 4.0-5.0 ml/h. The capillary tubing was joined to glass tubing 38 cm long and 5 mm in diameter. Two cm of high purity surgical grade latex rubber tubing connected the glass tubing to the reservoir flask at C.

The 250 ml Erlenmeyer culture flask (F) was stoppered with a cotton plug through which glass tubing was inserted. The liquid medium drained through an overflow tube at G, containing a ground glass fitting attached to a sintered glass filter. The culture flasks were agitated on a shaker rotating at 66 rpm. Four reservoir flasks supplying 16 culture flasks were used. Each culture flask overflowed at a volume of approximately 75 ml.

Figure 1. Diagram of a continuous culture apparatus for the growth of filamentous marine fungi. A, air inlet; B, reservoir flask, C, rubber tubing; D, capillary tubing; E, glass tubing; F, culture flask; G, exit tube with sintered-glass filter.

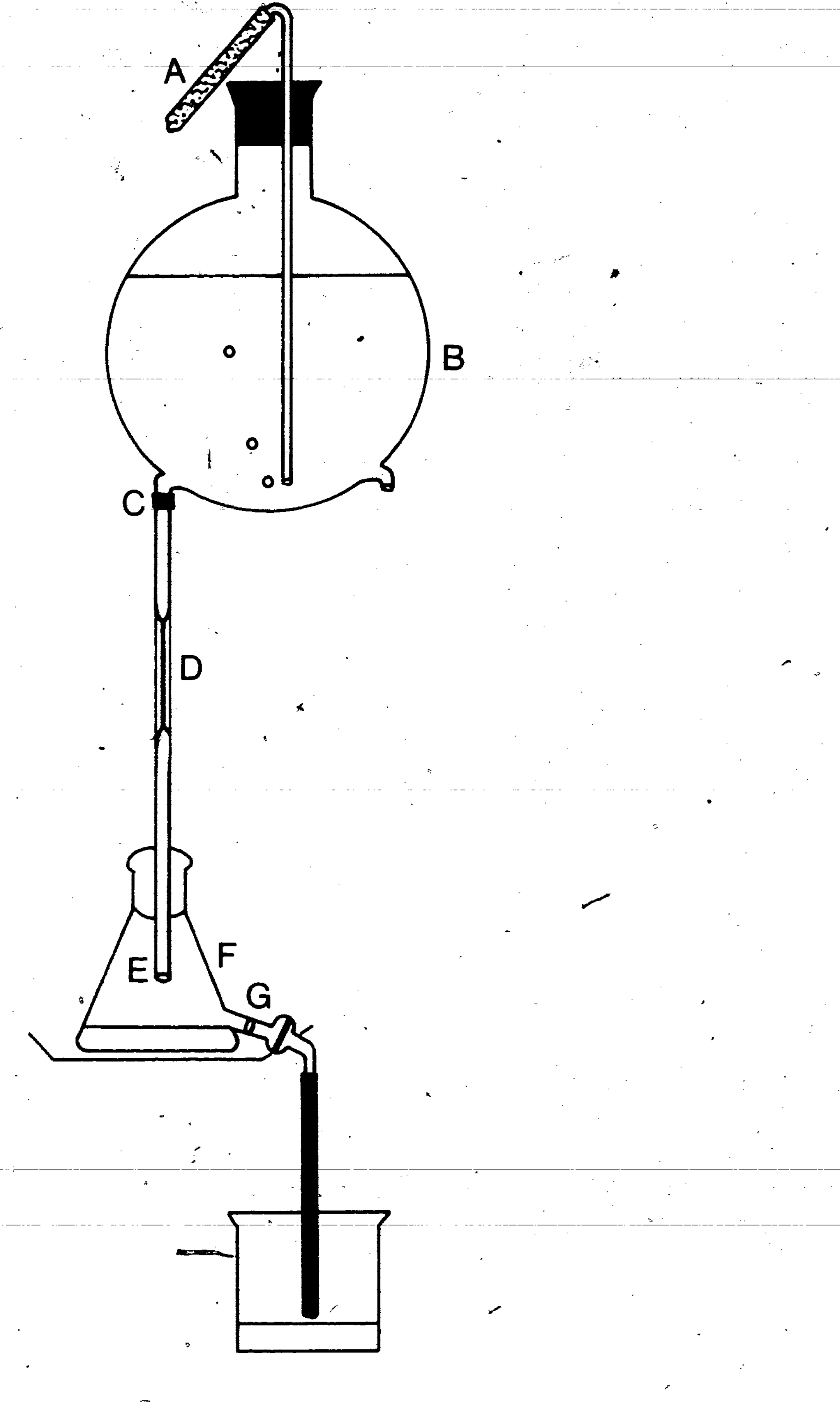
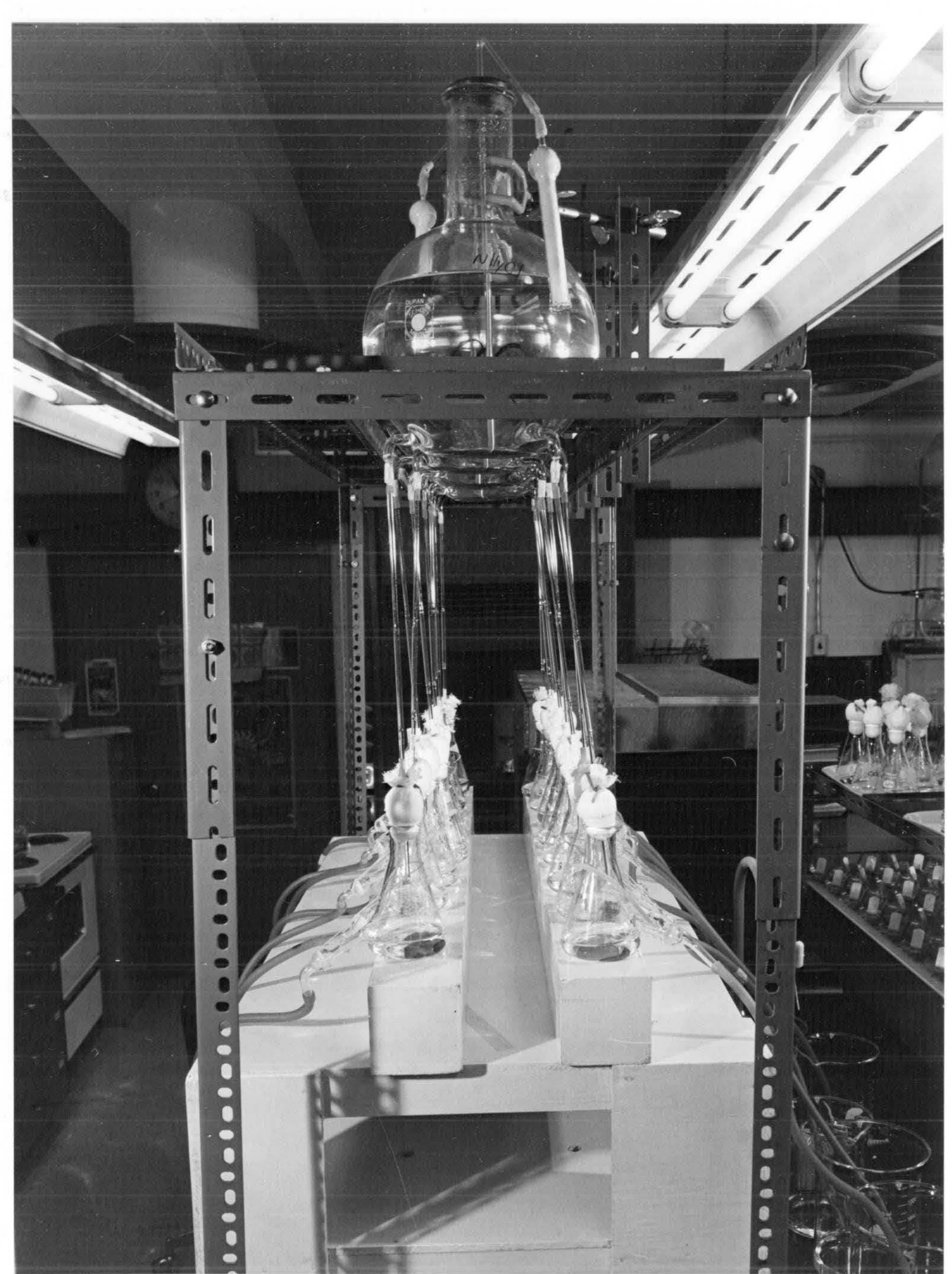

A
B
C
D
E
F
G

Figure 2. Photograph of a continuous culture apparatus for the growth of filamentous marine fungi.



Flow rates were monitored regularly by measuring the volume of liquid medium leaving the culture flasks.

Early designs of the continuous culture apparatus substituted surgical latex rubber tubing for the glass tubing used in the present model. It was subsequently determined that all types of natural and synthetic tubing tested were toxic to one or all of Z. maritimum, H. alopallonella, and M. pelagica. Lengths of sulfur-free, surgical latex, and silicone rubber tubing were shaken in nutrient solution for 2 weeks and removed; growth was assessed on these and control solutions. The toxic property left in the nutrient solution by these tubings inhibited growth. Subsequently, the amount of tubing used was reduced to the minimum necessary for adequate flexibility. Currently, no more than 5 mm of tubing is exposed to the nutrient flow.

E. Comparative growth in continuous and batch culture

The nutrient solution for the continuous culture apparatus was prepared as follows. Five liters of artificial seawater containing 7.2 g THAM were adjusted with 1N HCl to pH 7.0 and autoclaved; all other NSA components were adjusted to pH 7.5 and filter sterilized. Autoclaving versus filter sterilization of artificial seawater did not affect growth of the three species. Both solutions were then added aseptically to the autoclaved reservoir flask. The final pH of the nutrient solution was approximately 7.7. The nutrients in the

reservoir flasks stood for several days to ensure lack of contamination. The contents of the continuous culture flasks were checked periodically for contamination by inoculation into 2216E medium (Zobell, 1946). The nutrient solution for the batch culture flasks was prepared as described in (B) above. The batch culture flasks used in the comparative growth curve experiments contained 75 ml of liquid medium.

The continuous culture flasks were inoculated with 4 mg of inoculum 3 days before nutrient flow was begun, allowing a pellet to be formed. For the comparative growth curves, three flasks from batch and continuous systems were removed every 4 days for dry weight determination. Mycelium from each flask was filtered, weighed, and the pH of the filtrate determined.

F. Expeiments with reinoculated media

Zalerion maritimum, H. alopallonella, and M. pelagica were grown in NSA for 2 weeks. The "old" nutrient solution was then filtered and divided into two portions; one portion was adjusted to pH 7.5 with 1N NaOH, the other portion was left at the low pH value produced by the growth of the fungus. These pH adjusted and unadjusted solutions were then divided into two portions, and fresh nutrients added to one portion of each. Twenty-five ml of these four solutions, artificial seawater, and NSA were added to 125 ml flasks, inoculated with 2 mg inoculum, incubated for 2 weeks, and the dry weight measured.

G. Buffer experiments

The basal nutrient solution (NSA) was made up with .005 M, .01 M, .02 M, .03 M, .04 M, and .05 M THAM and MES (2-[N-morpholino] ethanesulfonic acid) respectively. Flasks containing these buffers were inoculated, dry weight measured after 1 and 2 weeks, and the pH of the filtrate measured.

H. Nutrient Uptake Experiments

Two isolates of Z. maritimum were grown in NSA containing 0.02 M MES instead of THAM. The medium was analyzed every 2 days for glucose, ammonium, phosphate, nitrate, and nitrite. Six flasks of each isolate were removed from the shaker; three flasks were used for dry weight and pH measurement. Three flasks were filtered and the filtrate analyzed for the abovementioned nutrients.

Analyses of all nutrients except ammonium were preceded by a dilution of the medium with seawater or distilled water. To obtain calibration curves NSA was made up at a range of nutrient concentrations and diluted acordingly.

After a 1:3 dilution with ASW, glucose was measured using orthotoluidine (Hultman, 1959). The absorption of the samples was measured at 610 nm with a Bausch & Lomb Spectronic 20

spectrophotometer. Nitrate and nitrite were measured according to Strickland and Parsons (1972). The samples were diluted 1:200 with ASW, and absorption measured on a Spectronic 20 at 543 nm. Phosphate was determined using the technique of Strickland and Parsons (1972). Samples were diluted 1:200 with distilled and deionized water, and absorption measured with a 10 cm cell at 885 nm on a Beckman DU spectrophotometer.

Ammonium was measured usng a gas-sensing ammonium electrode (Orion Model 95-10) coupled to a Fisher Accumet model 520 digital pH/ion meter. After alkalification of the nutrient solution, ammonium ion was converted to ammonia gas which diffused through the differentially permeable probe membrane.

The terms "more" or "less", when used in the text to modify growth or ion uptake, mean significantly more or significantly less at the 95% probability level. The statistical test used was either the student T-test or the Student Newman Keul multiple range test, dependent upon whether comparisons were made between two or several means.

RESULTS

A. Growth of two isolates of *Zalerion*

Figures 3A and 3B show photographs of the two isolates after growth for 3 weeks at 20 C on seawater agar. Notable differences were: the olive pigmentation in isolate FMC vs. grey pigmentation in Isolate H 135, and the more distinct formation of radial zones by the FMC isolate.

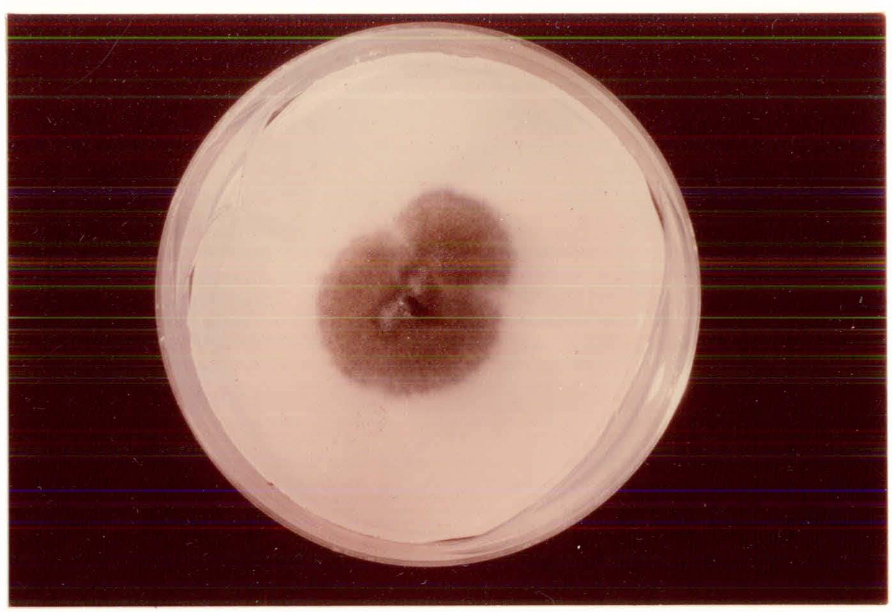
B. The continuous culture system

The apparatus (Figs. 1-2) was operated for 20 days or longer without contamination or significant change in flow rates. The removable sintered glass exit tubes did not clog with mycelium during the 21 day incubation period, and were not replaced.

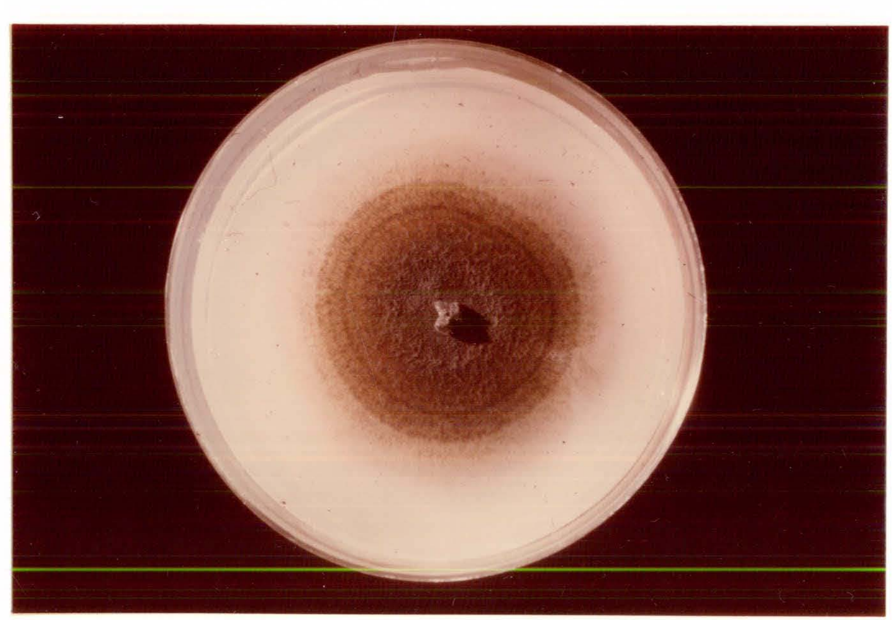
C. Growth in continuous and batch systems

The growth rates of each species (Fig. 4) were higher in continuous than batch culture. In the case of *Z. maritimum* there was a five-fold difference in dry weight after 21 days. In batch culture, pieces of mycelium which became separated from the main pellet did not grow. In the continuous system mycelium continued growing on the bottom and sides of the flasks. Occasionally more than one large

Figure 3. Growth of _Z. maritimum_ H 135 and FMC on seawater agar. A, FMC; B, H 135.



A

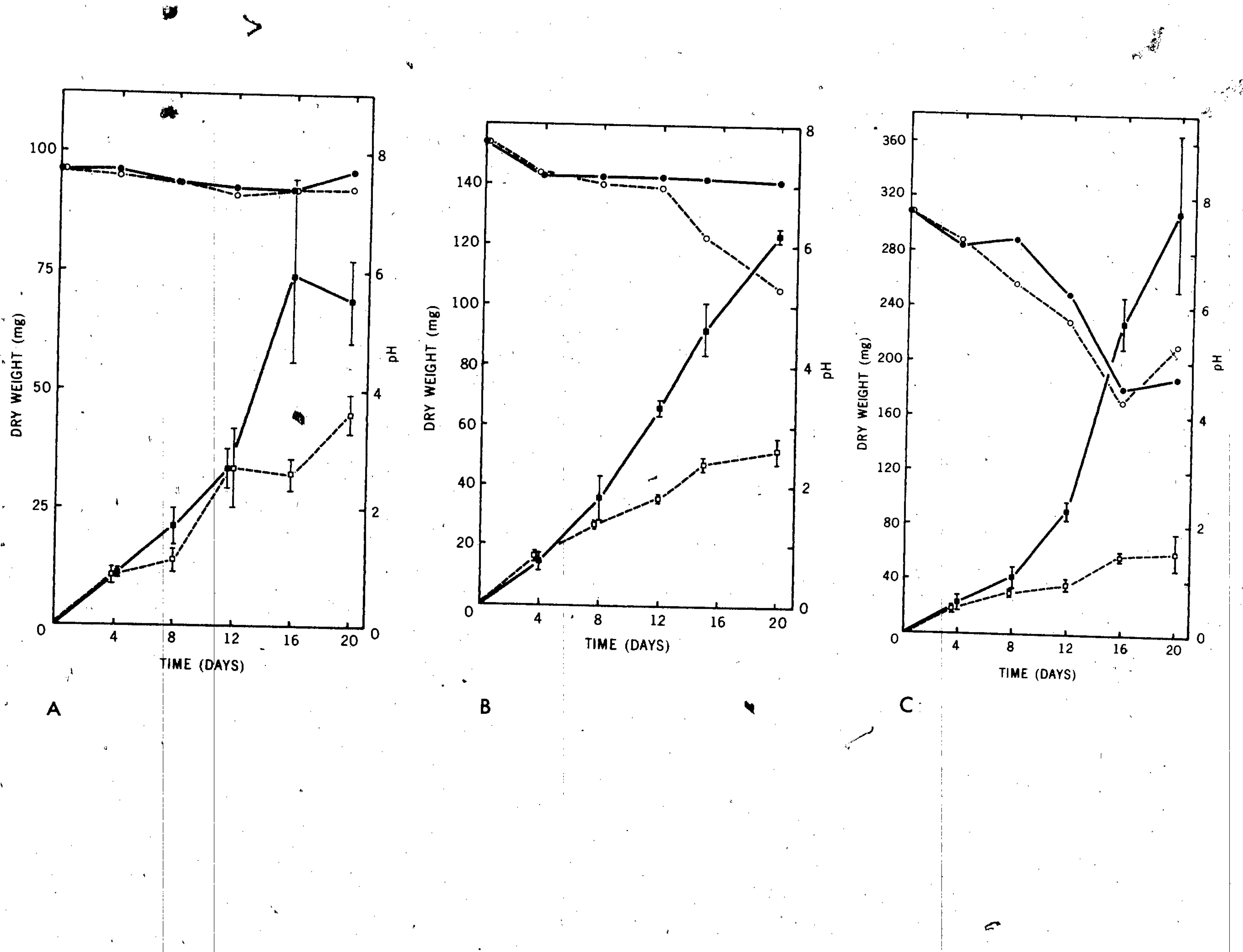


B

Figure 4. Growth of *M. pelagica* (A), *H. alopallonella* (B), and *Z. maritimum* (C) in continuous and batch culture.

□ - growth, batch culture ■-growth, continuous culture

○ - pH, batch culture ●-pH, continuous culture


DRY WEIGHT (mg)
pH
TIME (DAYS)
A
DRY WEIGHT (mg)
pH
TIME (DAYS)
B
DRY WEIGHT (mg)
pH
TIME (DAYS)
C

pellet would be formed. In Z. maritimum the appearance of mycelium grown in the two systems differed. After 8 days in batch culture, the mycelial pellet was dark olive to black and sporulating heavily. After 8 days in continuous culture, the mycelial pellet was light, diffuse, and produced very few spores. After 21 days, the mycelium nearly filled the continuous culture flask, and had darkened in certain areas.

When a mycelial pellet of Z. maritimum FMC was transferred to a continuous flask after several weeks' incubation in a closed flask, a halo of actively growing, light coloured mycelium developed around the pellet within 48 hours.

The nutrient solution was not buffered by THAM when Z. maritimum FMC was grown in either continuous or batch culture (Fig. 4). Growth of H. alopallonella and M. pelagica in continuous culture was lower than growth of Z. maritimum. There was no difference in pigmentation or sporulation of the mycelium in either species. During growth of H. alopallonella, the pH of the batch culture solution fell to 5.6, whereas the pH of the continuous culture solution fell to 7.0. The pH of both solutions remained constant during growth of M. pelagica.

D. Experiments with reinoulated media

After 2 weeks' growth in NSA, Z. maritimum FMC had lowered the pH

of the nutrient solution to 4.2. Growth was poor on reinoculated, low pH media (Table I). More growth occurred on low pH media with than without added nutrients. Growth on pH adjusted, reinoculated media was as high as on NSA. There was a greater pH drop after growth on pH adjusted media with than without added nutrients. The mycelium growing in low pH flasks was beige in colour, whereas the mycelium growing in all other solutions was black.

Growth of M. pelagica (Table I) was poor in reinoculated, low pH media (pH 5.5). Growth was slightly increased by the addition of nutrients. Growth of M. pelagica in pH adjusted, reinoculated media was greater than in NSA. There was a greater pH drop in pH adjusted media with than without added nutrients.

Growth of H. alopallonella was lowest on low pH (5.4) medium without added nutrients (Table I). Growth on all other solutions was equal, except for the higher growth on pH adjusted media with added nutrients.

E. Buffer experiments

At all concentrations MES buffered more effectively than THAM (Table II). Growth on media containing MES was slightly lower than growth on media containing THAM. Each compound buffered more effectively at higher concentrations. The higher concentrations of each buffer did not reduce growth.

Table I. Growth of *Z. maritimum* EMC, *M. pelagica* and *H. alopallonella* in batch culture on reinoculated media.

Solution	*Z. maritimum* Dry weight (mg)	*Z. maritimum* Final pH	*M. pelagica* Dry weight (mg)	*M. pelagica* Final pH	*H. alopallonella* Dry weight (mg)	*H. alopallonella* Final pH
Artificial seawater	2.9* $\pm$0.3**	8.2	4.7 $\pm$0.2	7.8	2.8 $\pm$0.1	8.1
No pH adjustment No added nutrients	15.9 $\pm$0.5	3.3	14.8 $\pm$0.6	3.1	18.9 $\pm$0.7	3.4
No pH adjustment Nutrients added	23.2 $\pm$0.7	2.9	18.2 $\pm$0.4	3.1	29.3 $\pm$1.3	3.2
NSA	35.5 $\pm$3.2	3.3	22.1 $\pm$0.7	3.4	28.3 $\pm$2.8	4.1
pH adjusted to 7.5; no added nutrients	39.3 $\pm$2.0	6.9	38.8 $\pm$ 2.7	5.5	29.0 $\pm$2.3	3.2
pH adjusted to 7.5; nutrients added	37.4 $\pm$1.5	4.6	28.5 $\pm$ 1.0	3.0	39.2 $\pm$2.9	5.1

Note: Means joined by a common line are not significantly different (Newman-Keuls test, $p < 0.05$)

* = mean, ** = standard error sample size = 4

Table II. Effect of different concentrations of THAM and MES buffers on growth of *Z. maritimum* FMC in batch culture.

Concentration (M)	Dry Weight (mg) TRIS $\bar{x}$	TRIS $\bar{sx}$	MES $\bar{x}$	MES $\bar{sx}$	Final pH TRIS	Final pH MES
Incubated 7 days						
0.005	34.0	1.6	27.5	3.5	3.7	6.5
0.01	37.9	5.0	29.8	1.5	4.1	6.8
0.02	32.9	2.3	26.6	0.7	6.0	7.0
0.03	39.9	1.6	30.3	0.4	6.8	7.1
0.04	31.9	0.4	26.8	1.2	7.0	7.2
0.05	31.9	13.4	30.7	1.3	7.1	7.3
Incubated 14 days						
0.005	58.4	13.6	51.4	1.4	3.1	6.2
0.01	50.9	2.4	45.1	0.7	3.4	6.6
0.02	51.8	1.3	47.7	4.5	5.1	7.0
0.03	54.8	1.5	46.0	5.2	5.3	7.0
0.04	54.3	4.6	50.3	6.5	5.9	7.1
0.05	53.7	2.4	44.4	2.5	6.4	7.3

Note: $\bar{x}$ = mean, $\bar{sx}$ = standard error, sample size = 3

F. Nutrient uptake experiments

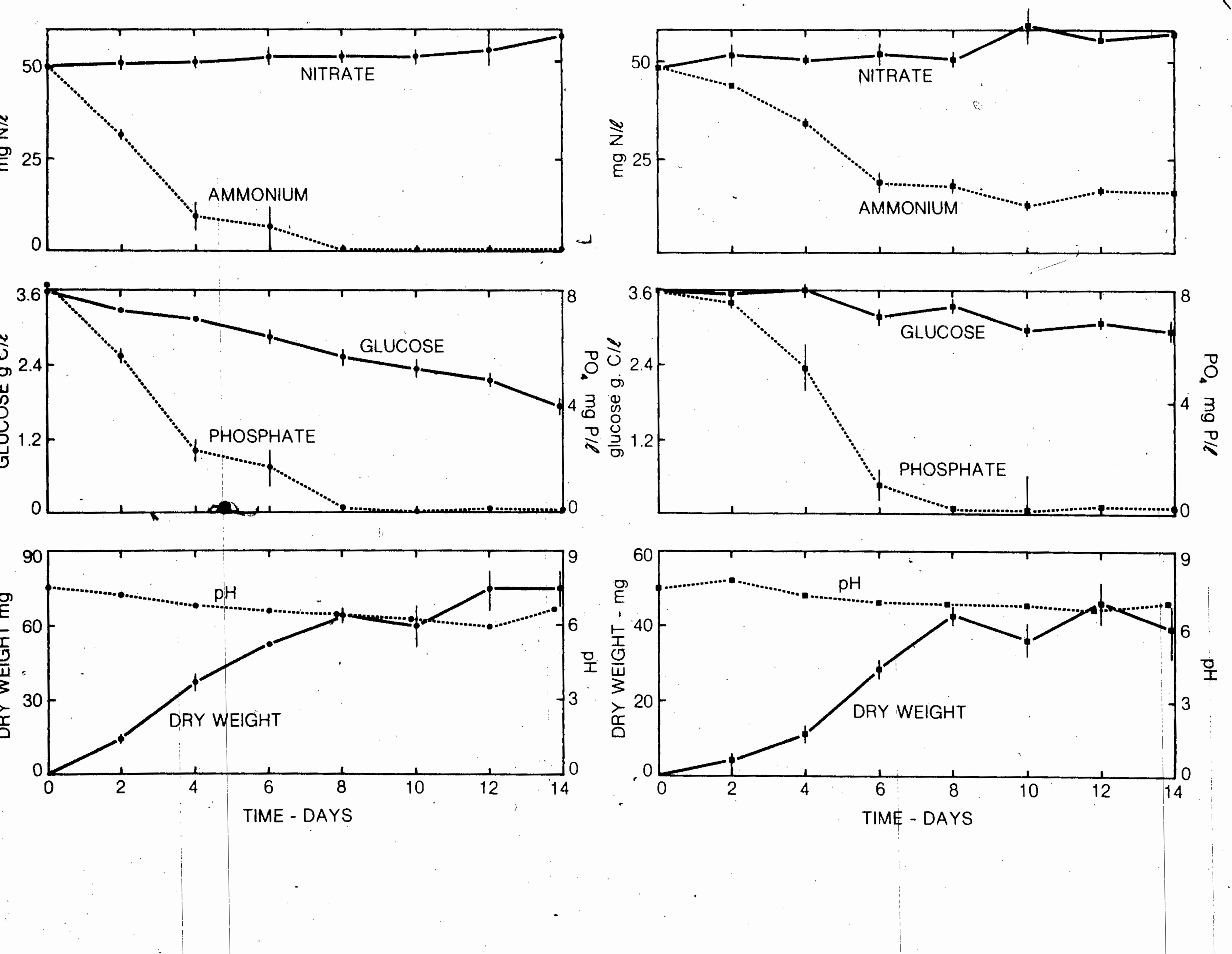
When MES was substituted for THAM, the pH did not fall below 6.0 during growth of _Z. maritimum_ FMC (Fig. 5). After 2 weeks' incubation, 50% of the glucose remained in the nutrient solution. Ammonium but not nitrate was removed rapidly during growth. Evaporation from the shake culture flasks was approximately 11% in the 2 week incubation period, and may account for the apparent increase in nitrate concentration. Phosphate was rapidly removed and was present in trace amounts after 8 days. A similar pattern of nutrient uptake was shown by Isolate H 135. After 14 days, 85% of the glucose remained in the nutrient solution. Ammonium was rapidly removed up to but not beyond day 6. Nitrate was not removed from the medium and apparently increased in concentration over the 14 day incubation period. As in the FMC isolate, trace amounts of phosphate were detected after 8 days. During growth of each isolate the medium was analyzed for the presence of nitrite, and none was found.

DISCUSSION

Many filamentous marine fungi can easily be grown in axenic culture on seawater yeast extract medium. Yeast extract is frequently included in both liquid and solid media as a source of phosphorus and vitamins (Sguros and Simms, 1963). The growth of marine fungi in chemically defined media is poor in comparison to media containing

Figure 5. Changes in dry weight, pH, and concentrations of glucose, phosphate, ammonium, and nitrate during growth in batch culture of *Z. maritimum* FMC and H 135.

● - FMC, ■ - H 135



NITRATE
AMMONIUM
mg N/ℓ
50
25
0
GLUCOSE
PHOSPHATE
GLUCOSE g C/ℓ
glucose g. C/ℓ
PO₄ mg P/ℓ
3.6
2.4
1.2
0
8
4
pH
DRY WEIGHT
DRY WEIGHT mg
DRY WEIGHT - mg
90
60
40
30
20
9
6
3
TIME - DAYS
2
4
6
8
10
12
14

yeast extract. Some investigators have substituted yeast extract ash for yeast extract (Sguros and Simms, 1964; Sguros et al,1973). The chemically defined nutrient solution used in these experiments was that of Sguros and Simms (1963), in which a mixture of vitamins and minerals was substituted for yeast extract.

The moderate growth rates shown by Z. maritimum, H. alopallonella, and M. pelagica on the chemically defined medium suggested a different system of cultivation. In order to increase growth rates and approximate the conditions of the marine environment, a continuous culture system was designed for the cultivation of marine fungi.

In the continuous culture system, nutrients flowed continuously over a mycelial pellet which was retained in a culture vessel. Zalerion maritimum, H. alopallonella, and M. pelagica were suited to cultivation in this apparatus because the mycelial fragments usually formed a single submerged pellet soon after inoculation. The continuous culture system was not a chemostat, in which steady state growth conditions within the culture flask are maintained by a constant efflux of homogeneous material. Although yeasts and some filamentous fungi have been grown in chemostats, the clumping and wall growth shown by these marine species would make steady state growth conditions difficult to achieve.

The use of 250 ml culture vessels allowed a comparison with batch culture results. However, if a higher biomass were required (i.e., should the marine fungi produce compounds of economic or biomedical importance), a larger culture vessel could be designed.

The flow rate of aproximately 4.5 ml/h was chosen to minimize media additions to the reservoir flasks to one per 14 day period. In addition to the inconvenience of making large volumes of media, frequent opening of the reservoir flask increased the risk of contamination. The flow rate could easily be lessened or increased by respectively lengthening or shortening the capillary tubing.

It was impossible to analyze the growth curves obtained in either batch or continuous culture according to any given mathematical function. Terms borrowed from bacterial growth kinetics do not necessarily describe fungal growth. Because growth of a filamentous fungus is not autocatalytic, the "exponential growth phase" of the bacterial growth curve is best termed the "phase of rapid growth" (Mandels, 1965) or the "linear growth phase" in the fungal growth curve. Mandels (1965) has suggested that a cube root function describes mathematically the growth of a fungal pellet in shake culture. However, the pellets of Z. maritimum, H. alopallonella, and M. pelagica were too variable for the use of a cubical function. Moreover, pieces of mycelium could separate from the main pellet and attach to the sides or bottom of the culture vessel, forming new centers for growth.

In continuous culture, growth continued throughout the 21 day incubation period. The growth rate of each species declined in continuous culture after day 16, possibly because of pH changes or because the fungus had expanded to fill the culture vessel. In batch culture, the duration of linear growth was dependent upon cultural conditions. In *Z. maritimum*, the most rapid growth was generally between 4 and 8 days (see growth curves in Chapter II), followed by a period of fluctuation in dry weight. Sguros *et al* (1962) partially attributed dry weight fluctuation in *Trichocladium achrasporum* (Meyers and Moore) Dixon to autolysis, and stated that dry weights beyond 8 days probably reflected the presence of dead and partially autolysed cells. In *Z. maritimum* linear growth could continue from 10 to 12 days or longer. In most experiments, growth measurements in batch culture were taken after 7 and 14 days. The 7 day measurements should represent the dry weight of actively growing mycelium, whereas the 14 day measurements may partially represent the accumulation of polysaccharides and other storage products in the older hyphae. In the continuous system, all experiments were harvested after 14 days, during the period of most rapid growth of each species tested.

The use of a continuous culture system showed a relationship between pigment production, sporulation, and cessation of growth in *Z. maritimum*. A pH change in the nutrient solution did not explain the onset of sporulation; mycelium in reinoculated, low pH media produced few spores. Cochrane (1958) stated that the major

stimulation to reproduction in the fungi was nutrient exhaustion. Perhaps after several days in batch culture and 16 days in continuous culture sporulation was stimulated by a lack of one or more nutrients.

Experiments were carried out to explain the different growth rates in continuous and batch culture. Pellets which had stopped growing in a closed flask grew again when transferred to the continuous culture system. Thus growth did not slow down in batch culture due to a determinate size factor, as may occur in certain other fungi (Gottlieb, 1971). This result also showed that growth was not inhibited by an intramycelial staling factor, which could be transported from the older hyphae to the periphery of the colony (Molitoris, 1974). Experiments with reinoculated media demonstrated that there were no non volatile staling products present in the nutrient solution. Only in *H. alopallonella* did nutrient exhaustion appear to be a growth inhibiting factor.

A pH effect may explain the poorer growth shown by *Z. maritimum*, *H. alopallonella*, and *M. pelagica* in batch culture. When ammonium is used as a nitrogen source, the uptake of this ion and the accompanying exchange of hydrogen ions from the cell generally results in a pH drop in the nutrient solution (Sguros and Simms, 1963). The poor growth of these three species in acidic media was improved by the addition of nutrients, which probably raised the pH or improved the buffering capacity of the media.

Growth of Z. maritimum and M. pelagica caused a greater pH drop in reinoculated, pH adjusted media with added nutrients, than in pH adjusted media without added nutrients. There was no growth difference to cause this result. A possible explanation is that in the solution without added nutrients, the ammonium was largely utilized during the first growth period. When the medium was reinoculated, the fungus used nitrate in addition to ammonium. The pH therefore did not fall as drastically as in the media which had received a fresh supply of NH_4NO_3. A pH drop in a nutrient solution containing NH_4NO_3 is generally considered presumptive evidence for the preferential utilization of ammonium (Nicholas, 1965).

It is clear that THAM did not buffer the nutrient solution effectively. For this reason MES, described as a biological buffer by Good et al (1966) and used by Child, Knapp, and Eveleigh (1973) to buffer agar media during the growth of several fungal species, was substituted for THAM in certain experiments. Growth was slightly lower on MES than on THAM; however, the former compound buffered more effectively at all concentrations tested.

The question was next asked whether one or more nutrients would limit growth in batch culture if the pH of the nutrient solution were controlled. Glucose was not exhausted, and although a large part (H 135) or all (FMC) of the ammonium was removed, a large supply of

nitrate remained in the medium. Studies with Scopulariopsis brevicaulis (Sacc.) Bainier (Morton and MacMillan, 1954) indicate that after exhaustion of ammonium, nitrate can be used. Both isolates had removed all of the phosphate after 8 days growth, at which time there was a marked decrease in the growth rate. A similar test with H. alopallonella and M. pelagica showed that they also removed the major portion of the phosphate from the nutrient solution.

Growth did not stop in batch culture simply because of phosphorus limitation. Increasing the amount of phosphorus in the medium did not markedly increase growth of either isolate. Possibly phosphate was removed from the nutrient solution and stored in an internal pool, as is the case with amino acids (Nicholas, 1965) and ammonia (Hunter and Segel, 1971). Siegenthaler et al (1967) demonstrated an intracellular phosphate pool in the marine Phycomycete Thraustochytrium roseum Goldstein. If this were the case in Z. maritimum, phosphorus would not necessarily limit growth despite its absence from the nutrient solution. In conclusion, the lower growth in batch culture was due to changes in pH of the nutrient solution. When the pH was controlled, the results suggest that lack of phosphorus, possibly in combination with other nutrients, limited growth in batch culture.

Since marine fungi in nature do not affect the pH or chemical composition of the surrounding nutrient solution, the continuous system may more closely approximate the marine environment than the

batch system. The continuous system may allow the use of dilute media, thus more accurately recreating marine nutrient concentrations. Despite these advantages, the continuous system should not replace the batch system in all future nutritional studies. Problems of making large volumes of media, limited sample size, and size of apparatus make this logistically impossible. Additionally, growth of each mycelial fragment in continuous culture results in large dry weight variations. I suggest that the continuous system be used in addition to the batch system, when interference in growth from nutrient exhaustion, pH changes, or staling products is suspected.

In summary, the moderate growth of _Z. maritimum_, _H. alopallonella_, and _M. pelagica_ in chemically defined media suggested the use of a continuous culture system. The significantly higher growth of all species in the continuous system led to an investigation of growth dynamics in the batch system. Analyses of nutrients in the culture medium and experiments with reinoculated media suggested that pH changes and depletion of phosphorus and/or other nutrients limited growth in batch culture. These results may allow the formulation of more balanced culture media for future nutritional studies.

The information on cultivation and growth which is presented in Chapter I provides the necessary background for an understanding of Chapter II, a study of nitrogen nutrition in _Zalerion maritimum_.

CHAPTER II

Nitrogen Nutrition in Zalerion maritimum

INTRODUCTION

Various studies have been carried out on the nitrogen requirements of terrestrial fungi. Many species (Sarbhoy, 1965; Said and Harhash, 1966; Agnihotri and Vaartaja, 1967; Mitra and Tandon, 1970) use both inorganic and organic nitrogen. Some fungi (Grover and Sidhu, 1965; Jones, 1965; Yusef and Allam, 1967; Stephen and Chan, 1970) grow best on organic nitrogen. Certan Phycomycetes cannot metabolize either nitrate or nitrite (Bhargava, 1945; Dayal, 1961; Barr, 1969, Nolan, 1970). Most fungi, when supplied with ammonium and nitrate, will preferentially utilize the former (Morton and MacMillan, 1954; Nicholas, 1965; Cove and Pateman, 1969). Ammonium and nitrite may be taken up simultaneously (Morton and MacMillan, 1954). The optimal nitrogen source and carbon to nitrogen ratio is not necessarily the same for growth and for sporulation (Yusef and Allam, 1967; Aube and Gagnon, 1969; Singh and Tandon, 1970).

The fungi do not play the same role as the bacteria in the terrestrial nitrogen cycle. There is no evidence that the fungi either fix atmospheric nitrogen (Benemann and Valentine, 1972) or denitrify inorganic compounds to nitrogen gas (Nicholas, 1965). Eylar

and Schmidt (1959) and Hatcher and Schmidt (1971) have shown that soil fungi such as *Aspergillus flavus* Link ex Fr. convert organic nitrogen compounds to nitrate. The main role of the fungi in the terrestrial nitrogen cycle is to break down organic molecules, and convert them to simpler and more utilizable chemical forms.

Little work has been done on the nitrogen requirements of marine fungi. Gustafsson and Fries (1956) studied growth of *Pleospora purpurascens* R. Sant., *Zalerion maritimum* and *Diplodia orae- maris* Linder on asparagine, $(NH_4)_2HPO_4$, and KNO_3. Johnson *et al* (1959) studied radial growth of several higher marine fungi on agar made up with various nitrogen sources and at various carbon to nitrogen ratios. Sguros and Simms (1963) studied the effect of various concentrations of carbon and nitrogen on growth of *Trichocladium achrasporum*, *Humicola alopallonella*, *Orbimyces spectabilis* Linder, and *Halosphaeria mediosetigera* (Cribb & Cribb). Holligan and Jennings (1972b) investigated the effect of nitrogen sources on carbohydrate metabolism in *Dendryphiella salina* (Suth.) Pugh and Nicot. Sguros *et al* (1973) carried out the most comprehensive study to date of nitrogen nutrition in the filamentous marine fungi. They grew *Halosphaeria mediosetigera*, *Humicola alopallonella*, and *Trichocladium achrasporum* on four inorganic nitrogen sources and 34 organic nitrogen sources, including amino acids, amines, and nucleic acids. Each was supplied singly as the nitrogen source and growth measured by dry weight determination.

Sguros et al (1973) also investigated the effects of varying concentrations of NH_4NO_3 on growth of these three species. Peters et al (1975) determined the mycelial free amino acid content of ten species of higher marine fungi.

Studies on the nitrogen requirements of marine Phycomycetes were carried out by Vishniac (1955), Goldstein (1963 a,b,c,), Goldstein and Belsky (1964), and Alderman and Jones (1971).

All of the abovementioned studies were carried out either on solid agar or in liquid, batch culture. In this study a continuous culture system was used in addition to the batch culture technique. It was hoped that a continuous culture system would more closely aproximate the conditions of the marine environment. Additionally, it was hoped that a comparison between the two systems would show whether cultivation techniques could affect nutritional results.

Previous investigators have supplied nitrogen sources singly in the growth medium. In their natural environment marine fungi are supplied with various nitrogen sources, available from both the cellulosic substrate and the surrounding seawater. In this study the relative removal from the nutrient solution of combinations of nutrients was determined by chemical analyses.

All of the nutritional experiments in this chapter were carried out with Zalerion maritimum, chosen because of its common occurrence in temperate marine waters and its possible importance as a wood decomposer (Jones, 1957-58). Because of the physiological variability of this fungus, two isolates were used for the majority of the experiments.

The goals of the experiments described in this chapter were as follows: to determine the effect of growing inoculum on various nitrogen sources on subsequent patterns of nitrogen nutrition; to determine what inorganic and organic nitrogen sources were utilized, and to compare these results with the availability of nitrogen in the sea; to determine which, if any, nitrogen sources were preferentially removed from the nutrient solution; to determine how much nitrogen was necessary for growth, and how changes in C:N ratio affected growth. Additionally, an attempt was made to determine whether Z. maritimum could carry out any of the conversions of the marine nitrogen cycle.

MATERIALS AND METHODS

Techniques of inoculum preparation, methods of cultivation, and the basal nutrient solution (NSA) were as described in Chapter I unless otherwise specified. All nitrogen sources were supplied at a concentraton of 100 mg N/l unless otherwise stated. Sample sizes for each experiment were indicated on the corresponding figures and graphs.

A. Growth of inoculum on various nitrogen sources

Portions of mycelium 5 mm in diameter of Z. maritimum FMC were removed from F 1003 agar plates and placed in NSA. NH_4NO_3 was replaced by NH_4Cl, $NaNO_2$, $NaNO_3$, or l-alanine. After 43 days growth the four batches of mycelium were macerated, quantified, and the inoculum adjusted to 4 mg/ml. Ten flasks containing each of the four nitrogen sources were inoculated with mycelium grown on the corresponding nitrogen source. After 7 days in shake culture, the mycelium was macerated, centrifuged, and washed. Inoculum grown on NH_4Cl was used to inoculate 10 flasks containing NH_4Cl, 10 flasks containing $NaNO_2$, 10 flasks containing $NaNO_3$, and 10 flasks containing l-alanine. After 7 and 14 days in shake culture, the mycelium was weighed and the pH of the nutrient solution measured. Inoculum grown on $NaNO_2$, $NaNO_3$, and l-alanine was treated in the same manner.

B. Growth on inorganic nitrogen sources

Growth of two isolates of Zalerion maritimum on various nitrogen sources was compared in continuous and batch culture. The NH_4NO_3 in NSA was substituted by $NaNO_3$, $NaNO_2$, NH_4Cl, or l-glutamic acid. Each of the four reservoir flasks of the continuous system contained one of the nitrogen sources. The culture flasks were inoculated 3 days before nutrient flow was begun. The culture flasks

from the continuous system were removed for dry weight measurement 14 days after inoculation. The pH of the effluent from the continuous system was measured every 2 days. Mycelial dry weight and pH in the batch culture flasks was measured after 1 and 2 weeks' growth.

The effect of different nitrogen concentrations on growth of Z. maritimum in batch and continuous culture was tested. NH_4NO_3 in NSA was substituted by 100 mg N/l, 10 mg N/l, 1 mg N/l, and 0.1 mg N/l of $NaNO_3$. Each of the reservoir flasks contained one of these solutions, and a fifth reservoir flask contained no nitrogen. The culture flasks were inoculated 3 days before nutrient flow was begun, and dry weight measured 14 days after incubation. The pH of the effluent was measured every 2 days. The dry weight and pH of the batch culture flasks was measured after 7 and 14 days.

The effect of various carbon and nitrogen concentrations on growth in continuous and batch culture was tested. NSA was prepared with the following concentratiions of glucose and $NaNO_3$: glucose 3.6 g C/l, $NaNO_3$ 100 mg N/l; glucose 0.36 g C/l, $NaNO_3$ 10 mg N/l; glucose 0.036 g C/l, $NaNO_3$ 1 mg N/l; glucose 0.0036 g C/l, $NaNO_3$ 0.1 mg/l. The experiment was carried out in batch and continuous culture using identical techniques to the experiment described above.

C. Growth on organic nitrogen sources.

1. Single amino acids

Growth of Z. maritimum FMC and H 135 was tested in batch culture in NSA to which 21 amino acids and $NaNO_3$ were added singly as sole nitrogen sources. Two mg of inoculum were added to 125 ml flasks containing 25 ml of liquid medium. After 7 days in shake culture, the mycelium was weighed and the final pH of the nutrient solution measured.

2. A combination of amino acids

L-arginine-HCl, l-alanine, and l-glutamic acid were added singly and in combination to NSA in continuous and batch culture. The single amino acids were added at a concentration of 100 mg N/l. The three amino acids were added equally to give a total concentration of 100 mg N/l. Dry weight was measured after 14 days in continuous culture, and the pH of the effluent was measured every 2 days. Dry weight and pH of the nutrient solution was measured in batch culture after 7 and 14 days.

3. Organic compounds other than amino acids

Both isolates were grown in batch culture on NSA containing urea, peptone, allantoic acid, allantoin, vitamin free casamino acids, and citrulline as sole sources of nitrogen. Nitrate was included for comparison with an inorganic nitrogen source. Bacto-peptone and vitamin-free casamino acids were added at concentrations of 0.6173 g/l

and 1.4285 g/l respectively. These amounts corresponded to approximately 100 mg N/l (Difco manual). Dry weight of mycelium and pH of the nutrient solution was measured after 7 and 14 days incubation.

D. Removal of nitrogen source combinations from the nutrient solution

1. Ammonium and nitrite

Isolates FMC and H 135 were grown in NSA containing 50 mg N/l each of $NaNO_2$ and NH_4Cl. In this experiment, two times the usual phosphate concentration of .06 g/l was added. MES was substituted for TRIS at a concentraton of .02 M. At 2 day intervals, three flasks were removed for dry weight and pH measurements. Three additional flasks were removed for determination of glucose, ammonium, nitrite, nitrate, and phosphate. The techniques for the analyses of these ions were described in Chapter I.

2. L-glutamic acid and nitrate

Both isolates were grown in NSA containing 50 mg N/l each of l-glutamic acid and $NaNO_3$. MES was substituted for TRIS at a concentration of .02 M. Every 2 days three flasks were removed for dry weight and pH measurements. Three additional flasks were removed for measurement of nitrate and glutamic acid. Nitrate was measured according to Strickland and Parsons (1972). To measure glutamic acid,

the nutrient solution was diluted 2:1 with ASW. One ml of ninhydrin solution (100 ml 4N sodium acetate buffer, 300 ml filtered methyl cellosolve, 8 g ninhydrin, 0.16 g $SnCl_2$) was added to 5 ml of dilute nutrient solution, mixed on a vortex mixer, and boiled for 30 seconds (Shriner, Fuson, and Curtin, 1964). After cooling, the absorbance of the samples was measured against an ASW blank on a Bausch and Lomb Spectronic-20 spectrophotometer at 570 nm. The results were compared with a calibration curve obtained by adding ninhydrin to various concentrations of glutamic acid in NSA. A control of uninoculated NSA was included in each analysis.

3. Ammonium and eight amino acids

Isolates FMC and H 135 were grown, in batch culture, in NSA which contained NH_4Cl and eight amino acids (l-aspartic acid, l-threonine, l-serine, l-glutamic acid, l-alanine, l-valine, l-isoleucine, and l-leucine). TRIS was replaced by .02 M MES. The eight amino acids were each added at .05 mM/l. Equimolar concentrations of the amino acids were used in order to obtain peaks of a similar height from the amino acid analyzer. For each amino acid, .05 mM/l corresponded to approximately 14 mg N/l. Nine flasks were removed from the shaker after 3,6,9, and 12 days. Three flasks were used for dry weight and pH measurements. The contents of three flasks were filtered (Millipore HA, 0.45 μ) and ammonium measured in the filtrate. Ammonium was measured according to Strickland and Parsons (1972). Absorbance was measured on a Beckman DU spectrophotometer at 640 nm.

Each sample was diluted 1:200 with distilled water and results compared with a calibration curve. The calibration curve was obtained by preparing a range of concentrations of NH_4Cl in NSA, followed by a 1:200 dilution with distilled water.

The final three flasks were removed every 3 days for extraction and analyses of the eight amino acids. Forty ml from each flask were filtered and frozen at -20 C. At the end of the experimental period, all test flasks plus three uninoculated control flasks were freeze dried in a Virtis Unitrap freeze-dryer, Model No. 10-100. The flasks were then desalted using the solvent extraction technique of Smith (1960). Samples were extracted three times with 10 ml of acetone containing 5 % 6N HCl. One ml of distilled water was added, the sample placed in ice, and the acetone blown off with forced air. Ten ml of distilled water were added and the samples refrigerated at 4 C. Amino acid concentrations were determined by adding 0.1 ml of sample to a Beckman Model 119 automatic amino acid analyzer. Those samples containing low concentrations of amino acids were added in quantities >0.1 ml. Three flasks of uninoculated medium were frozen, extracted, and added to the amino acid analyzer as standards. The accuracy of the column data was ±10-15%. The analyses took 3 weeks, after which the amino acid concentrations of the control flasks were remeasured.

E. Conversions of nitrogen

In order to determine whether Z. maritimum FMC and H 135 grew anaerobically, mycelium was transferred onto eight seawater agar plates. Four were placed in a Brewer anaerobic jar containing a BBL GasPak, and four were incubated in air. The GasPak generated sufficient H_2 and CO_2 to create an anaerobic environment. Both isolates were also grown in a candle jar.

1. Nitrogen fixation

The nitrogen fixation test was adapted from Postgate (1972). Both isolates were grown in test tubes containing NSA without nitrogen, with 15 g/l of agar. The slants were incubated in air and in a candle jar. After 2 and 6 weeks, the cotton plugs were pushed inside and serum stoppers placed on the end of each tube. Five cc of acetylene were injected into each tube, and into an uninoculated control. Gas samples were injected into a Carlo Erba Fractovap Model GV Gas Chromatograph, and the resultant peaks compared with standard acetylene and ethylene peaks. In case the addition of 5 cc of acetylene was toxic (acetylene would then constitute 25% of the gas volume), the experiment was repeated using 1 cc of acetylene.

2. Denitrification

Two methods were used to test for denitrification by Zalerion maritimum. Test tubes containing nitrate broth (Difco) were made up

with artificial seawater. Inverted Durham tubes were added and the test tubes were autoclaved. The tubes were inoculated with a heavy suspension of FMC or 135, tightly sealed with screw caps, and shaken such that the mycelium went inside the Durham tube. The tubes were observed for 4 weeks for formation of gas inside the Durham tube.

Additionally, nitrate agar (Difco) was made up with artificial seawater and poured into the bottom of test tubes. Stab cultures were then made of Isolates FMC and H 135. Cooled nitrate agar was poured overtop of the first agar layer. These cultures were observed for 4 weeks for any evidence of gas formation.

To test for formation of nitrite and ammonia from nitrate, FMC and H 135 were grown on NSA in which $NaNO_3$ was the sole nitrogen source. After 7 and 14 days in batch culture, three flasks of each isolate were removed, filtered, and the filtrate analyzed for nitrite and ammonium. Nitrite was measured according to Strickland and Parsons (1972) and ammonium was measured with the ammonium electrode.

3. Nitrification

FMC and 135 were grown, in continuous and batch culture, on NSA containing NH_4Cl as the sole nitrogen source. TRIS was replaced by .02 M MES. Batch culture flasks were removed after 7 and 14 days, filtered, and analyzed for nitrite and nitrate.

RESULTS

A. Growth of inoculum on various nitrogen sources

Table III shows the results of growing inoculum on a variety of nitrogen sources. Inoculum grown on l-alanine grew best on l-alanine after both 7 and 14 days incubation. Growth was less and equal on $NaNO_3$, $NaNO_2$, and NH_4Cl. Inoculum grown on NH_4Cl grew equally on all four nitrogen sources after 7 and 14 days. Inoculum grown on $NaNO_2$ grew best on $NaNO_2$ and l-alanine after 7 days. After 14 days, growth was higher on l-alanine than on the other three nitrogen sources. Inoculum grown on $NaNO_3$ grew best on l-alanine and $NaNO_3$ after one weeks' incubation. After 2-weeks' incubation, there was no difference in growth between the four nitrogen sources.

B. Growth on inorganic nitrogen sources.

1. NH_4Cl, $NaNO_3$, $NaNO_2$, and l-glutamic acid

Both isolates of Z. maritimum utilized each of l-glutamic acid, NH_4Cl, $NaNO_2$, and $NaNO_3$ in continuous and batch culture (Tables IV-V). Growth of both isolates was highest on the organic nitrogen source after 1 weeks' incubation in batch culture. After 2 weeks in batch culture, growth of Isolate FMC was highest on the organic nitrogen source, and growth of Isolate H 135 was equal on all nitrogen sources. Growth of isolate FMC was higher in batch culture after 1

Table III. The effect of growing inoculum in various nitrogen sources on growth of *Z. maritimum* FMC in batch culture.

Nitrogen source	Grown on NH_4Cl One week Dry weight (mg)	Grown on NH_4Cl One week Final pH	Grown on NH_4Cl Two weeks Dry weight (mg)	Grown on NH_4Cl Two weeks Final pH	Nitrogen source	Grown on $NaNO_2$ One week Dry weight (mg)	Grown on $NaNO_2$ One week Final pH	Grown on $NaNO_2$ Two weeks Dry weight (mg)	Grown on $NaNO_2$ Two weeks Final pH
$NaNO_3$	49.8* ± 4.3**	7.8	65.7 ± 10.1	7.3	$NaNO_3$	40.0 ± 0.8	7.9	61.7 ± 2.6	7.8
$NaNO_2$	54.0 ± 2.5	7.8	65.9 ± 11.4	7.6	NH_4Cl	45.3 ± 2.1	3.6	74.0 ± 2.9	3.2
NH_4Cl	58.0 ± 2.8	6.8	59.4 ± 8.5	6.5	$NaNO_2$	52.3 ± 3.9	7.9	72.7 ± 4.5	7.9
L-alanine	61.6 ± 2.3	7.5	80.8 ± 16.4	6.7	L-alanine	56.4 ± 5.1	8.0	93.7 ± 5.1	7.7

Nitrogen source	Grown on L-alanine One week Dry weight (mg)	Grown on L-alanine One week Final pH	Grown on L-alanine Two weeks Dry weight (mg)	Grown on L-alanine Two weeks Final pH	Nitrogen source	Grown on $NaNO_3$ One week Dry weight (mg)	Grown on $NaNO_3$ One week Final pH	Grown on $NaNO_3$ Two weeks Dry weight (mg)	Grown on $NaNO_3$ Two weeks Final pH
$NaNO_3$	55.2 ± 4.5	8.0	66.4 ± 15.1	8.0	NH_4Cl	38.1 ± 1.1	3.4	48.5 ± 3.0	3.3
$NaNO_2$	45.0 ± 7.3	7.8	73.3 ± 5.6	8.0	$NaNO_2$	41.8 ± 3.7	7.8	71.9 ± 8.9	7.8
NH_4Cl	49.0 ± 3.4	3.5	73.3 ± 1.5	3.1	$NaNO_3$	62.2 ± 0.6	7.6	63.8 ± 12.4	7.8
L-alanine	74.6 ± 6.1	7.9	127.3 ± 4.5	7.8	L-alanine	57.1 ± 1.8	7.7	65.4 ± 6.4	7.3

Note: Means joined by a common line are not significantly different (Newman-Keuls test, $p < 0.05$)
* = mean, ** = standard error sample size = 4

Table IV. The effect of inorganic nitrogen and l-glutamic acid on growth of _Z. maritimum_ FMC in continuous and batch culture.

Nitrogen source	CONTINUOUS		BATCH			
			One week		Two weeks	
	Dry weight (mg)	Final pH	Dry weight	Final pH	Dry weight	Final pH
$NaNO_3$	224.9* ± 38.4**	6.3	45.3 ± 1.1	7.8	66.0 ± 4.7	8.0
$NaNO_2$	197.2 ± 20.8	7.3	42.5 ± 1.8	7.5	73.8 ± 2.4	7.9
NH_4Cl	253.4 ± 31.9	5.0	54.1 ± 3.9	4.3	73.9 ± 2.4	3.5
L-glutamic acid	180.8 ± 21.0	7.0	55.1 ± 2.5	7.7	95.5 ± 3.7	7.9
No nitrogen	39.6 ± 0.8	7.3	13.8 ± 0.5	7.5	17.5 ± 0.5	7.6

Note: Means joined by a common line are not significantly different (Newman-Keuls test, $p < 0.05$)

* = mean, **=standard error sample size = 4

Table V. The effect of inorganic nitrogen sources and L-glutamic acid on growth of Z. maritimum H 135 in continuous and batch culture.

Nitrogen source	CONTINUOUS		BATCH			
			One week		Two weeks	
	Dry weight (mg)	Final pH	Dry weight (mg)	Final pH	Dry weight (mg)	Final pH
$NaNO_3$	99.6* $\pm$ 4.5**	7.5	32.6 $\pm$ 0.5	7.7	54.0 $\pm$ 13.0	7.6
$NaNO_2$	128.5 $\pm$ 21.7	7.5	29.1 $\pm$ 1.9	7.7	51.9 $\pm$ 2.5	7.5
NH_4Cl	104.1 $\pm$ 2.2	7.4	35.3 $\pm$ 2.1	6.3	52.7 $\pm$ 4.0	6.5
L-glutamic acid	96.1 $\pm$ 3.2	7.7	53.0 $\pm$ 10.0	7.1	71.3 $\pm$ 5.1	7.5
No nitrogen	27.9 $\pm$ 2.3	7.4	12.6 $\pm$ 0.5	7.5	14.9 $\pm$ 0.7	7.6

Note: Means joined by a common line are not significantly different (Newman-Keuls test, $p < 0.05$)

* = mean, ** = standard error sample size = 4

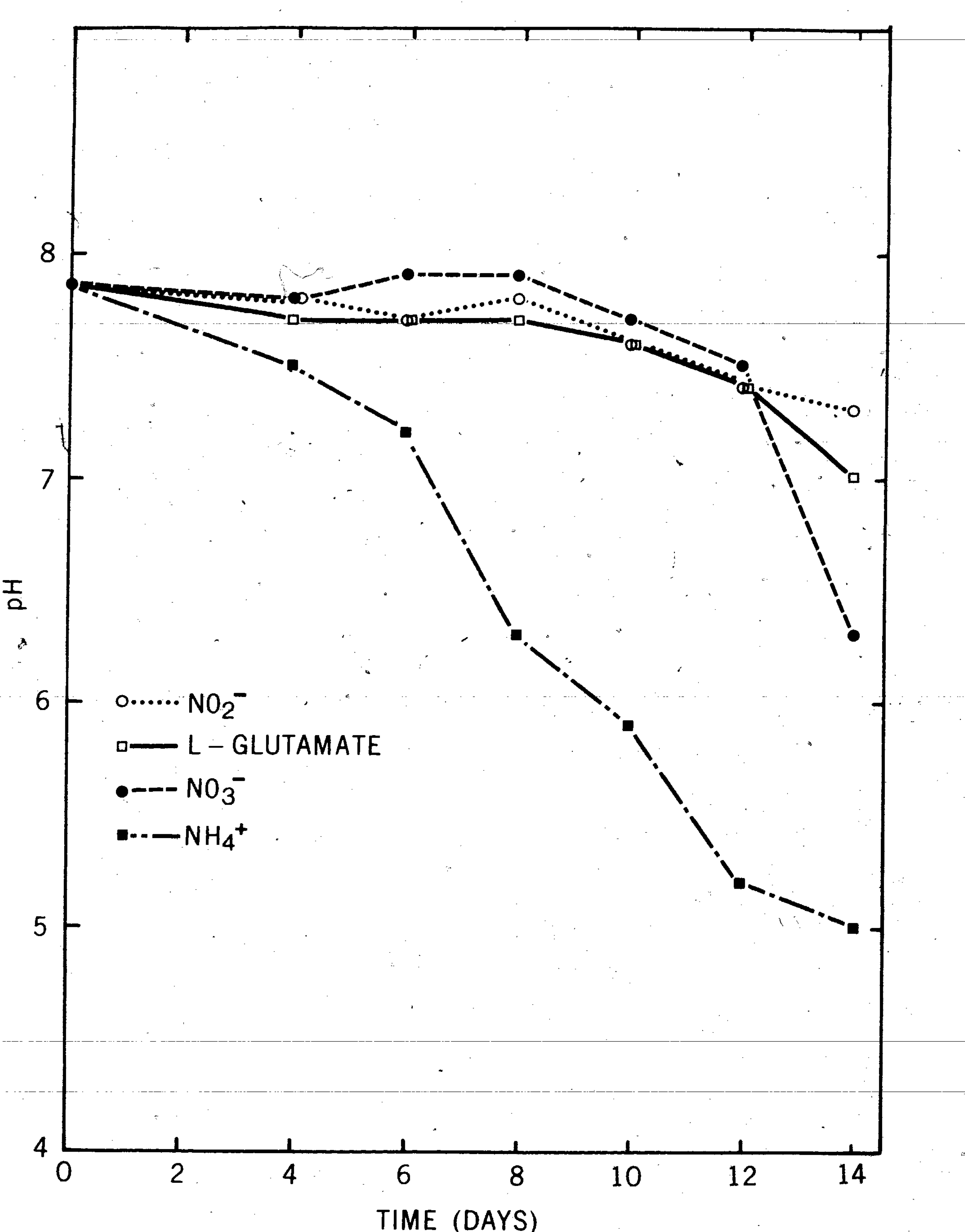
week in the ammonium containing media than in media containing nitrate or nitrite. After 2 weeks' incubation, growth was equivalent on the three inorganic nitrogen sources. Growth of both isolates lowered pH in batch culture medium containing ammonium. In batch culture, the pH of media containing nitrate, nitrite, or l-glutamic acid was raised by growth of Isolate FMC.

In continuous culture, there was no difference in growth on the various nitrogen sources. Isolate H 135 grew less in 2 weeks than did FMC and caused little pH change in the continuous culture medium. There was a steady pH drop (Fig. 6) when FMC was grown in continuous culture on ammonium. When isolate FMC was grown without nitrogen in continuous culture the mycelium became very black and sporulated heavily. This contrasted with the light coloured, asporogenous mycelium in all other flasks.

2. Various concentrations of nitrate

In both continuous and batch culture, growth of Isolate FMC (Table VI) was equal at 100 mg N/l and 10 mg N/l. There was no difference in growth, in continuous and batch culture, between flasks with 1 mg N/l, 0.1 mg N/l, and no nitrogen. Growth in continuous culture was higher than growth in batch culture at all concentrations tested. Growth was also tested (unpublished results) in NSA containing 200 mg N/l. Growth in continuous and batch culture was equal at 200 mg N/l and 100 mg N/l. The lower the concentration of

Figure 6. Changes in pH during growth of *Z. maritimum* FMC in continuous culture.



pH
8
7
6
5
4
0
2
4
6
8
10
12
14
TIME (DAYS)
NO2⁻
L – GLUTAMATE
NO3⁻
NH4⁺

Table VI. The effect of different concentrations of nitrate on growth of *Z. maritimum* FMC in continuous and batch culture.

Concentration mg N/l	CONTINUOUS		BATCH			
			One Week		Two weeks	
	Dry weight (mg)	Final pH	Dry weight (mg)	Final pH	Dry weight (mg)	Final pH
100	147.2* ± 23.4**	5.6	45.5 ± 6.6	7.8	60.3 ± 11.0	7.8
10	142.6 ± 20.7	6.7	40.4 ± 5.7	7.4	56.1 ± 5.5	7.6
1	40.4 ± 6.4	7.3	20.0 ± 0.7	7.4	30.0 ± 0.8	7.6
0.1	28.5 ± 3.3	7.4	14.2 ± 0.8	7.0	20.1 ± 0.1	7.1
0	34.2 ± 3.7	7.4	8.0 ± 1.4	7.4	17.0 ± 1.0	7.6

Note: Means joined by a common line are not significantly different (Newman-Keuls test, $p < 0.05$)

* = mean, ** = standard error sample size = 4

nitrogen, the darker the pigmentaton of mycelium in both culture systems.

Zalerion maritimum H 135 (Table VII) grew equally in continuous culture at 100 mg N/l and 10 mg N/l. In batch culture, growth was lower at 10 mg N/l than at 100 mg N/l after one weeks' incubation. Growth at 1 mg N/l, 0.1 mg N/l, and on NSA without nitrogen was equal in batch culture and equal in continuous culture. The pigmentation of the mycelium was light at all concentrations both in batch and continuous culture.

3. Various concentrations of nitrate and glucose

There was progressively lower growth of Z. maritimum FMC in batch and continuous culture as concentrations of nitrate and glucose were lowered (Table VIII). In batch culture, growth was equal at 1 mg N/l,, 0.036 g C/l and 0.1 mg N/l, 0.0036g C/l. At the two lowest concentrations, the pigmentation of the mycelium was very dark in both continuous and batch culture.

Zalerion maritimum H 135 (Table IX) grew as well in continuous culture at 100 mg N/l, 3.6 g C/l as at 10 mg N/l, 0.36 g C/l. Growth in continuous culture at 1 mg N/l, 0.03 g C/l was higher than growth at 0.1 mg N/l, 0.0036 g C/l. In batch culture, growth was greatest at the highest concentration of carbon and nitrogen. Growth was higher at 10 mg N/l, 0.36 g C/l than at 1 mg N/l, 0.036 g C/l and 0.1 mg N/l,

Table VII. The effect of different concentrations of nitrate on growth of *Z. maritimum* H 135 in continuous and batch culture.

Concentration mg N/l	CONTINUOUS		BATCH			
			One week		Two weeks	
	Dry weight (mg)	Final pH	Dry weight (mg)	Final pH	Dry weight (mg)	Final pH
100	100* ± 5.0**	7.5	42.7 ± 1.0	7.8	69.0 ± 1.8	7.7
10	90.4 ± 10.4	7.1	36.5 ± 3.6	8.1	59.0 ± 2.4	7.8
1	36.4 ± 6.9	6.9	21.0 ± 0.2	8.0	26.0 ± 0.24	7.9
0.1	43.2 ± 4.1	7.0	18.6 ± 0.8	8.1	22.5 ± 1.1	8.0
0	28.0 ± 2.0	7.6	18.1 ± 0.8	8.0	22.0 ± 1.0	8.0

Note: Means joined by a common line are not significantly different (Newman-Keuls test, $p < 0.05$)

* = mean, ** = standard error sample size = 4

Table VIII. The effect of different concentrations of nitrate and glucose on growth of Z. maritimum FMC in continuous and batch culture.

Concentration	CONTINUOUS		BATCH			
			One week		Two weeks	
	Dry weight (mg)	Final pH	Dry weight (mg)	Final pH	Dry weight (mg)	Final pH
3.6 g C/l 100 mg N/l	214.2* $\pm$ 33.2**	6.3	36.8 $\pm$ 0.6	7.6	69.1 $\pm$ 4.9	7.7
0.36 g C/l 10 mg N/l	110.1 $\pm$ 4.9	5.8	21.9 $\pm$ 1.6	7.7	20.8 $\pm$ 1.0	7.8
.036 g C/l 1 mg N/l	34.9 $\pm$ 3.5	7.6	7.1 $\pm$ 0.4	7.8	4.7 $\pm$ 0.5	7.7
.0036 g C/l 0.1 mg N/l	10.2 $\pm$ 0.7	7.6	5.0 $\pm$ 0.8	7.7	4.5 $\pm$ 0.4	7.7

Note: Means joined by a common line are not significantly different (Newman-Keuls test, $p < 0.05$)

* = mean, ** = standard error sample size = 4

Table IX. The effect of different concentrations of nitrate and glucose on growth of Z. maritimum H 135 in continuous and batch culture.

Concentration	CONTINUOUS		BATCH			
			One week		Two weeks	
	Dry weight (mg)	Final pH	Dry weight (mg)	Final pH	Dry weight (mg)	Final pH
3.6 g C/l 100 mg N/l	139.3* ± 12.4**	6.6	40.8 ± 2.2	7.2	70.5 ± 1.1	7.2
0.36 g C/l 10 mg N/l	114.8 ± 6.8	6.5	32.0 ± 3.0	7.7	28.0 ± 1.5	7.5
0.036 g C/l 1 mg N/l	70.4 ± 5.1	7.5	5.4 ± 0.2	7.8	6.0 ± 0.6	7.7
.0036 g C/l 0.1 mg N/l	9.6 ± 2.6	7.8	3.1 ± 0.2	7.7	3.4 ± 0.5	7.7

Note: Means jointed by a common line are not significantly different (Newman-Keuls test, $p < 0.05$)

* = mean, **=standard error sample size = 4

0.0036 g C/l. Growth of both isolates was higher in continuous than batch culture at all concentrations tested.

C. Growth on organic nitrogen sources

1. Single amino acids

Although H 135 and FMC 1 grew to a limited extent on nearly all of the 21 amino acids tested (Table X), only a few supported good growth. Isolate H 135 grew best on l-valine, l-alanine, l-glutamic acid, l-aspartic acid, l-glutamine, l-asparagine and l-arginine. Isolate FMC grew best on l-alanine and l-arginine. Both isolates grew poorly on l-glycine, l-methionine, l-cystine, l-lysine, and l-histidine. A yellow green diffusible pigment was produced by Isolate FMC in those flasks which contained ring-containing amino acids.

2. A combination of amino acids

In continuous culture, both isolates grew as well on single amino acids as on the combination of amino acids. Growth of Isolate FMC (Table XI) after 7 days in batch culture was higher on l-alanine and the three amino acid mixture than on l-arginine and l-glutamic acid. After 14 days, growth was equal on all four nitrogen sources. In batch culture, pigments had formed after 7 days in the flasks containing l-arginine-HCl, l-glutamic acid, and the three amino acid mixture.

Table X. The effect of single amino acids on growth of Z. maritimum FMC and H 135 in batch culture.

Amino acid	Isolate FMC		Isolate H 135	
	Dry weight	Final pH	Dry weight	Final pH
Monoamino monocarboxylic				
L-glycine	12.1* ± 0.4**	7.8	11.5 ± 0.6	8.0
L-leucine	15.9 ± 2.3	7.3	16.0 ± 1.3	7.1
L-valine	17.0 ± 1.5	6.8	24.1 ± 1.3	7.1
L-isoleucine	16.0 ± 1.2	7.6	17.1 ± 0.5	7.4
L-alanine	26.0 ± 0.2	7.5	23.2 ± 3.2	7.1
L-proline	13.2 ± 0.3	7.7	17.1 ± 1.4	7.4
Monoamino dicarboxylic				
L-glutamic acid	14.3 ± 1.2	7.7	21.4 ± 0.5	7.5
L-aspartic acid	18.9 ± 0.8	7.9	23.7 ± 2.3	7.7
Diamino dicarboxylic				
L-glutamine	11.4 ± 0.7	7.7	19.1 ± 2.0	7.5
L-asparagine	8.5 ± 0.8	7.9	23.4 ± 5.9	7.5

Table X (continued)

Amino acid	Isolate FMC Dry weight	Isolate FMC Final pH	Isolate H 135 Dry weight	Isolate H 135 Final pH
Ring-containing				
L-tryptophan	13.8 $\pm$ 0.7	7.2	13.4 $\pm$ 2.5	7.4
L-tyrosine	17.4 $\pm$ 1.5	7.5	16.7 $\pm$ 1.0	7.6
L-phenylalanine	15.5 $\pm$ 0.1	7.2	15.6 $\pm$ 2.4	7.3
Sulfur-containing				
L-methionine	9.4 $\pm$ 0.6	7.5	11.9 $\pm$ 0.9	7.3
L-cystine	10.9 $\pm$ 0.9	7.7	11.5 $\pm$ 0.5	7.8
Basic				
L-arginine HCl	20.6 $\pm$ 1.1	5.8	24.2 $\pm$ 0.5	6.4
L-lysine	6.9 $\pm$ 1.3	7.7	12.1 $\pm$ 0.7	7.6
L-histidine	5.0 $\pm$ 0.7	8.0	13.2 $\pm$ 1.1	7.8
With hydroxyl groups				
L-serine	16.1 $\pm$ 1.6	7.5	14.4 $\pm$ 1.1	7.4
L-threonine	13.4 $\pm$ 0.7	7.5	13.6 $\pm$ 0.5	7.4
Other				
β-alanine	13.8 $\pm$ 0.2	7.3	13.0 $\pm$ 0.3	7.4

Table X (continued)

Amino Acid	Isolate FMC Dry weight	Isolate FMC Final pH	Isolate H 135 Dry weight	Isolate H 135 Final pH
No nitrogen	5.1 ± 1.4	7.4	7.7 ± 0.8	7.8
$NaNO_3$	11.7	7.9	20.8 ± 0.9	7.4

Note: * = mean ** = standard error sample size = 3

Table XI. The effect of a combination of amino acids on growth of *Z. maritimum* FMC in continuous and batch culture.

	CONTINUOUS		BATCH			
			One week		Two weeks	
Nitrogen source	Dry weight (mg)	Final pH	Dry weight (mg)	Final pH	Dry weight (mg)	Final pH
L-arginine-HCl	118.1* ± 13.8**	6.1	46.1 ± 5.1	7.1	83.7 ± 17.8	6.3
L-glutamic acid	106.6 ± 8.2	7.1	38.6 ± 3.3	7.7	85.4 ± 13.2	7.0
3 amino acids	104.5 ± 8.6	6.3	57.0 ± 4.0	6.7	96.7 ± 10.4	6.8
L-alanine	134.5 ± 10.2	5.3	62.5 ± 2.8	7.4	102.2 ± 6.7	7.3

Note: Means joined by a common line are not significantly different (Newman-Keuls test, $p < 0.05$)

* = mean, ** = standard error sample size = 4

Growth of Isolate H 135 (Table XII) was equal after 7 days in batch culture on all four nitrogen sources. After 14 days, growth was higher on l-arginine and l-alanine than on l-glutamic acid and the amino acid combination.

3. Growth on organic nitrogen sources other than amino acids

Zalerion maritimum FMC (Table XIII) grew on urea, peptone, vitamin free casamino acids, allantoin, allantoic acid, and citrulline. Growth was poorest on allantoic acid, best on urea. *Zalerion maritimum* H 135 (Table XIV) grew on urea, peptone, vitamin free casamino acids, allantoin, and citrulline. Growth on citrulline occurred during the second week but not the first week of incubation. Isolate H 135 grew best on urea and peptone.

D. Growth on combinations of nitrogen sources

1. Ammonium and nitrate

The results in Chapter I showed that both isolates of *Z. maritimum* took up ammonium in preference to nitrate. Nitrate was not used by either isolate during the 14 day incubation period.

2. Ammonium and nitrite

Zalerion maritimum FMC took up nitrite and ammonium simultaneously from the nutrient solution (Fig. 7). Ammonium was removed at a faster rate than nitrite. Phosphate was not exhausted

Table XII. The effect of a combination of amino acids on growth of _Z. maritimum_ H 135 in continuous and batch culture.

Nitrogen source	CONTINUOUS		BATCH One week		BATCH Two weeks	
	Dry weight (mg)	Final pH	Dry weight (mg)	Final pH	Dry weight (mg)	Final pH
Arginine-HCl	212.9* $\pm$ 13.4**	7.3	69.3 $\pm$ 8.4	6.6	121.1 $\pm$ 8.1	5.6
L-Alanine	171.2 $\pm$ 12.8	7.2	65.4 $\pm$ 6.8	6.8	107.7 $\pm$ 9.2	6.6
L-Glutamic acid	266.4 $\pm$ 50.5	5.2	54.4 $\pm$ 3.1	5.7	78.9 $\pm$ 6.5	5.7
3 amino acids	189.7 $\pm$ 25.0	6.3	50.1 $\pm$ 3.5	[illegible]	80.7 $\pm$ 2.3	5.1

Note: Means joined by a common line are not significantly different (Newman-Keuls test, $p < 0.05$)

* = mean, ** = standard error sample size = 4

Table XIII. The effect of organic nitrogen sources on growth of *Z. maritimum* FMC in batch culture.

Nitrogen source	One week: Dry weight (mg) $\bar{x}$	One week: Dry weight (mg) $\bar{sx}$	One week: Final pH	Two weeks: Dry weight (mg) $\bar{x}$	Two weeks: Dry weight (mg) $\bar{sx}$	Two weeks: Final pH
No nitrogen	17.8	1.5	7.7	25.5	0.4	7.5
Allantoic acid	25.3	1.3	7.6	35.1	0.7	7.4
L-citrulline	31.0	2.0	7.1	44.3	2.7	6.4
Vitamin-free casamino acids	37.7	7.0	4.9	58.4	6.4	4.7
Peptone	38.0	5.0	6.2	60.2	7.5	6.3
Nitrate	38.2	3.8	7.7	51.3	5.1	7.8
Allantoin	40.7	3.3	6.7	55.6	2.6	7.1
Urea	50.3	2.5	6.0	62.1	3.5	6.1

Note: Means joined by a common line are not significantly different (Newman-Keuls test, $p < 0.05$)

$\bar{x}$ = mean, $\bar{sx}$ = standard error sample size = 4

Table XIV. The effect of organic nitrogen sources on growth of Z. maritimum H 135 in batch culture.

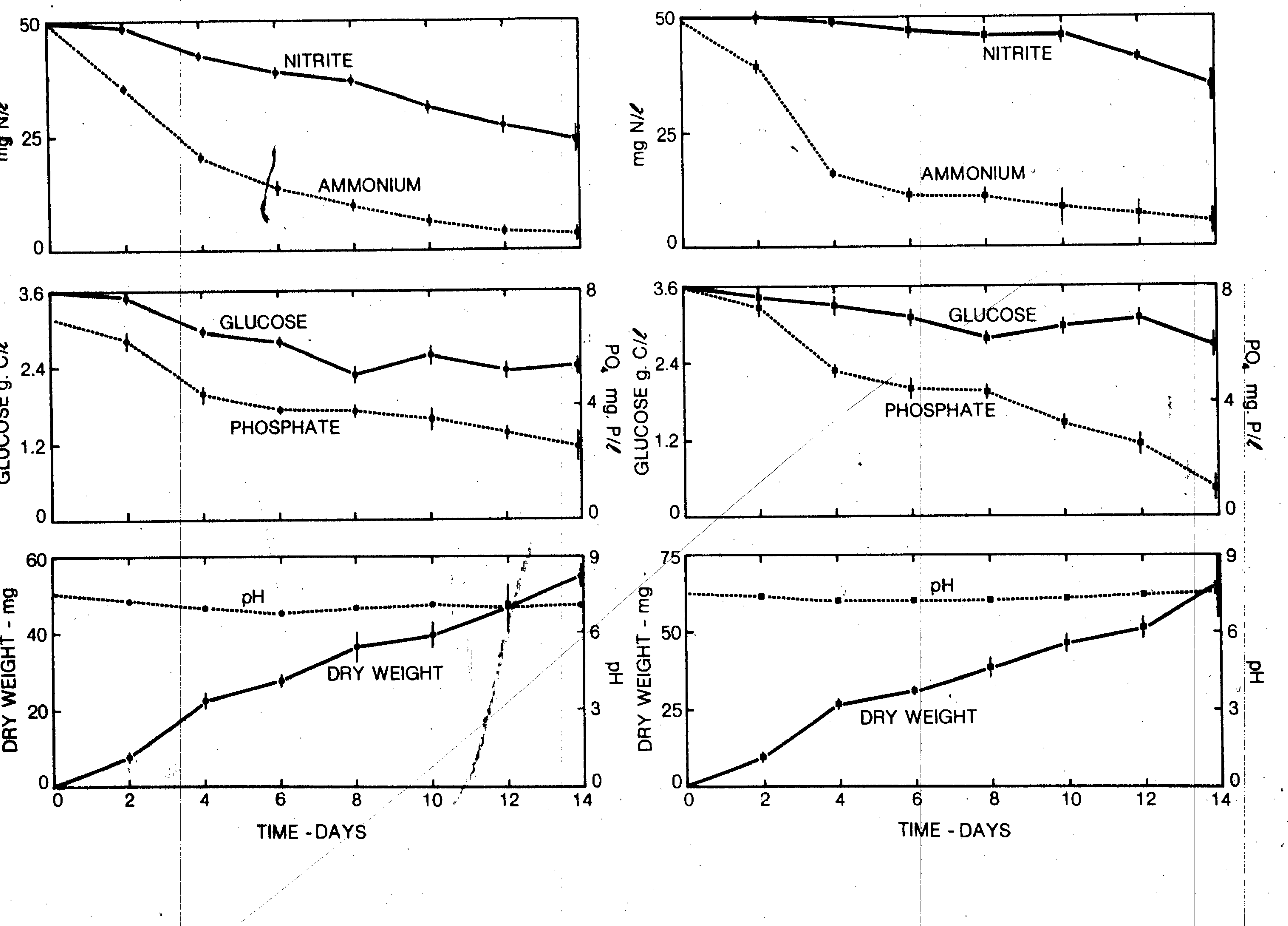
Nitrogen source	One week			Two weeks		
	Dry weight (mg)		Final pH	Dry weight (mg)		Final pH
	$\bar{x}$	$\bar{sx}$		$\bar{x}$	$\bar{sx}$	
No nitrogen	18.1	0.8	8.0	22.0	1.0	8.0
Allantoic acid	13.9	0.5	7.8	21.0	2.7	7.0
L-citrulline	14.5	0.5	7.6	52.9	13.5	7.0
Allantoin	20.1	0.6	7.5	62.6	7.7	7.3
Nitrate	31.2	2.4	7.5	50.7	2.7	7.5
Urea	31.7	1.3	7.4	73.6	7.1	6.1
Vitamin free casamino acids	36.6	0.5	6.2	63.4	5.6	5.3
Peptone	42.2	2.3	6.8	74.5	8.1	7.0

Note: Means joined by a common line are not significantly different (Newman-Keuls test, $p < 0.05$)

$\bar{x}$ = mean, $\bar{sx}$ = standard error sample size = 4

Figure 7. Changes in dry weight, pH and concentrations of glucose, phosphate, ammonium, and nitrite during growth in batch culture of _Z. maritimum_ FMC and H 135.

● - FMC, ■ - H 135.



mg N/ℓ
NITRITE
AMMONIUM
GLUCOSE g. C/ℓ
GLUCOSE
PHOSPHATE
PO4 mg. P/ℓ
DRY WEIGHT - mg
pH
DRY WEIGHT
TIME - DAYS
mg N/ℓ
NITRITE
AMMONIUM
GLUCOSE g. C/ℓ
GLUCOSE
PHOSPHATE
PO4 mg. P/ℓ
DRY WEIGHT - mg
pH
DRY WEIGHT
TIME - DAYS

during the 14 day incubation period. Isolate H 135 (Fig. 7) removed ammonium rapidly from the nutrient solution during the first 4 days of growth. Nitrite was not taken up until day 4, and was then slowly taken up for the duration of the experiment. After 14 days, 11% of the phosphate remained in the nutrient solution. Neither isolate excreted nitrate at any time during the experiment.

3. Nitrate and l-glutamic acid

The two isolates responded differently to the presence of these two nitrogen sources in the nutrient solution (Fig. 8). Isolate FMC preferentially took up the amino acid, exhausting it from the nutrient solution after 9 days. Nitrate was not removed from the nutrient solution during the 14 day incubation period. Isolate H 135 took up l-glutamic acid exclusively until day 6, whereupon the nitrate was removed, in smaller quantities, along with the l-glutamic acid.

4. Eight amino acids and ammonium

Zalerion maritimum FMC (Table XV) rapidly removed l-threonine, l-serine, l-alanine, l-valine, l-isoleucine, and l-leucine from the nutrient solution. The levels of l-glutamic acid and l-aspartic acid were comparatively high and variable throughout the experiment. Measureable concentrations of tyrosine and phenylalanine were detected after 9 and 12 days. Ammonium was rapidly removed during the first three days of incubation. The ammonium level then rose and stayed relatively constant for the duration of the experiment. Despite the

Figure 8. Changes in dry weight, pH, and concentrations of l-glutamic acid and nitrate during growth in batch culture of Z. maritimum FMC and H 135.

● - FMC, ■ - H 135.

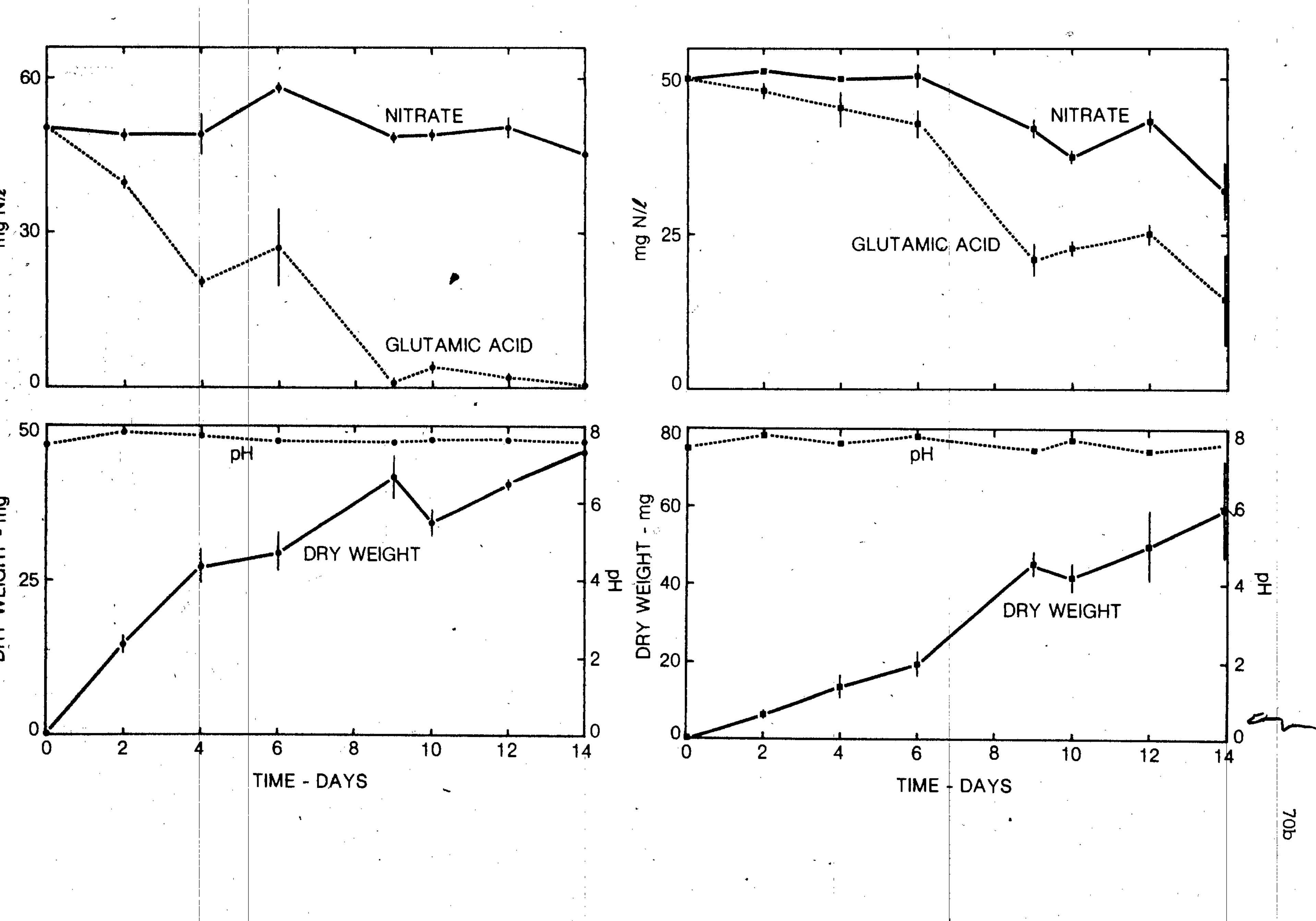

NITRATE
GLUTAMIC ACID
mg N/l
pH
DRY WEIGHT
DRY WEIGHT - mg
TIME - DAYS
NITRATE
GLUTAMIC ACID
mg N/l
pH
DRY WEIGHT
DRY WEIGHT - mg
TIME - DAYS

Table XV. Changes in concentrations of amino acids in the nutrient solution during growth of Z. maritimum FMC in batch culture.

Amino acid	Before Inoculation	Concentration µM Day 3	Day 6	Day 9	Day 12
Aspartic acid	50	40 ± 7.3	32 ± 9.7	44 ± 14.6	44 ± 9.5
Threonine	50	39 ± 2.9	28 ± 2.9	8 ± 3.5	4 ± 2.8
Serine	50	44 ± 5.3	33 ± 6.2	13 ± 6.0	1 ± 0.04
Glutamic acid	50	45 ± 4.4	42 ± 2.9	53 ± 13.1	30 ± 26.8
Alanine	50	37 ± 3.4	26 ± 2.3	3 ± 2.0	0
Valine	50	28 ± 1.7	11 ± 0.2	0	0
Isoleucine	50	30 ± 2.0	12 ± 0.6	0.4 ± 0.4	0
Leucine	50	30 ± 2.0	12 ± 0.6	0.5 ± 0.4	0.2 ± 0.2
NH_4Cl (mM)	3.56	1.39 ± 0.23	2.42 ± 0.30	2.58 ± 0.22	2.30 ± 0.43
Dry Weight (mg)		14.5 ± 0.1	33.9 ± 4.8	41.2 ± 4.3	45.2 ± 5.3
Final pH	7.5	7.3	6.4	6.3	5.9

Note: Numbers are means of three samples ± one standard error.

addition of MES, the pH fell to slightly below 6.0 during the experiment. Isolate H 135 (Table XVI) took up, to some extent, all of the amino acids supplied in the nutrient solution. L-aspartic acid, l-alanine, l-valine, l-isoleucine, and l-leucine were taken up at a faster rate than were l-threonine, l-serine, and l-glutamic acid. There was more variation in amino acid concentration measurements after 9 and 12 days incubation than there was after 3 and 6 days incubation. Isolate H 135 removed ammonium from the nutrient solution throughout the growth period.

E. Conversions of nitrogen sources

1. Nitrogen fixation

Both isolates grew on agar without nitrogen. Acetylene was not reduced by any of the samples tested.

2. Denitrification

When grown on nitrate agar and nitrate broth, there was no measureable N_2 production by either isolate. Neither isolate grew in the stab culture where the conditions were anaerobic.

When isolate FMC was grown in NSA containing nitrate, nitrite was detected in the nutrient solution after 1 and 2 weeks' growth. Nitrite was detected after 2 weeks' growth of Isolate 135. There was no measureable ammonium produced by either isolate. The ammonium

Table XVI. Changes in concentrations of amino acids in the nutrient solution during growth of Z. maritimum H 135 in batch culture.

Amino acid	Before Inoculation	Concentration μM			
		Day 3	Day 6	Day 9	Day 12
Aspartic acid	50	39 ± 1.1	26 ± 0.9	27 ± 6.6	25 ± 5.4
Threonine	50	39 ± 2.5	32 ± 1.6	27 ± 6.6	37 ± 1.4
Serine	50	40 ± 2.1	34 ± 1.3	32 ± 9.2	35 ± 2.2
Glutamic acid	50	42 ± 3.5	31 ± 1.0	34 ± 5.4	32 ± 4.0
Alanine	50	35 ± 2.7	29 ± 1.2	21 ± 9.0	26 ± 3.3
Valine	50	30 ± 2.7	20 ± 2.0	7 ± 5.9	11 ± 5.1
Isoleucine	50	32 ± 2.8	27 ± 2.3	9 ± 4.1	21 ± 8.3
Leucine	50	33 ± 2.7	25 ± 2.6	14 ± 7.4	27 ± 5.7
NH_4Cl (mM)	3.56	1.78 ± 0.89	1.17 ± 0.01	0.91 ± 0.17	0.85 ± 0.1
Dry Weight (mg)		16.5 ± 0.4	43.8 ± 3.9	61.3 ± 6.1	66.1 ± 4.0
Final pH	7.5	7.3	6.8	6.6	6.7

Note: Numbers are means of three samples ± one standard error.

concentrations in the inoculated solutions after 1 and 2 weeks' incubation were lower than in the uninoculated control.

3. Nitrification

Neither isolate excreted measureable amounts of nitrite or nitrate after 1 weeks' growth on NSA with NH_4Cl as a nitrogen source.

The observation was made that although Z. maritimum FMC did not grow anaerobically, growth on an agar plate incubated in a candle jar was greater than growth in air. After 3 weeks' incubation, the diameter of colonies grown in candle jars was approximately 15 mm greater than colonies grown in air.

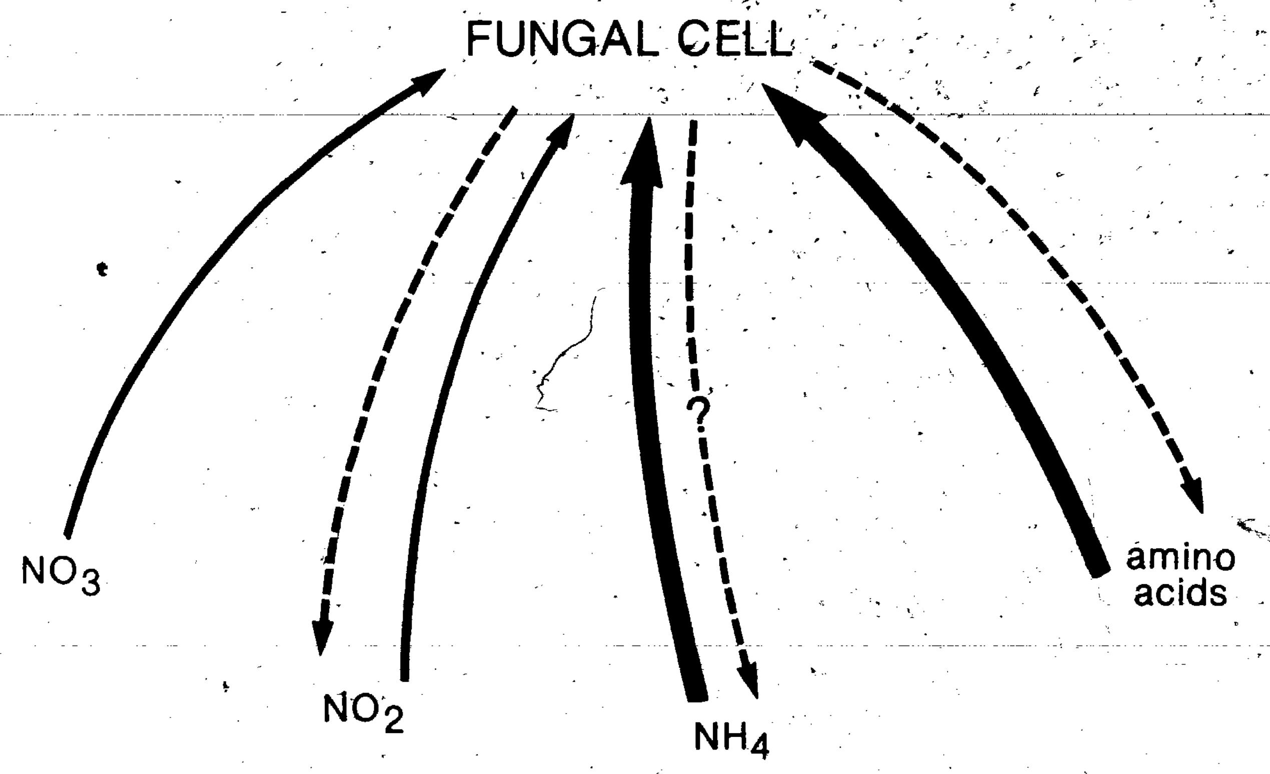
Figure 9 summarizes the hypothetical role of Z. maritimum in the marine nitrogen cycle.

DISCUSSION

Nitrogen nutrition in Z. maritimum will be discussed, followed by a comparison of these results with the availability of nitrogen in the sea.

The effect of growing inoculum on various nitrogen sources on patterns of nitrogen nutrition has not been investigated in marine

Figure 9. Summary diagram of nitrogen nutrition in *Z. maritimum*.



Nitrogen sources used for growth.
Preferred nitrogen sources for growth.
Nitrogen sources excreted into solution.

fungi. This study demonstrated that growth was partially dependent upon the nutritional history of the inoculum. This was evident after 1 week of incubation, before the mycelium had "acclimatized" to the nitrogen source. Despite the history of the inoculum, the organic nitrogen source (in this case alanine) generally supported the highest growth. Inoculum grown on $NaNO_2$ grew better on $NaNO_2$ than on the other inorganic nitrogen sources; inoculum grown on $NaNO_3$ grew better on $NaNO_3$ than on the other inorganic nitrogen sources. It is possible that the enzymes necessary for the reduction of nitrate and nitrite were induced when the inoculum was grown on the corresponding nitrogen source. There would therefore be no lag period necessary for the synthesis of nitrite and nitrate reductase when the inoculum was transferred to fresh medium. Inoculum grown in ammonium did not prefer ammonim to other inorganic nitrogen sources. Possibly the inoculum was affected by the low pH conditions produced by the uptake of this ion.

The results from the nitrogen utilization studies indicate that all three of the inorganic nitrogen sources commonly found in the sea (Vaccaro, 1965) support growth of Z. maritimum. This species can apparently use any one of nitrate, nitrite, or ammonia to synthesize the amino acids necessary for mycelial protein. Other investigators have also demonstrated that marine fungi can grow on inorganic sources of nitrogen. Sguros et al (1973) found that Halosphaeria mediosetigera, Trichocladium achrasporum, and Humicola alopallonella

grew on each of NH_4Cl, KNO_3, KNO_2, and NH_4NO_3. Johnson et al (1959) found that 21 species of higher marine fungi grew on agar containing $NaNO_3$, KNO_3, and $(NH_4)_3PO_4$. Tubaki (1966) showed that one marine fungus could utilize the end product of nitrogen reduction by another marine fungus. He demonstrated that Ceriosporopsis halima Linder could grow on nitrite containing media only when cultivated with Corollospora maritima Werdermann, the former species utilizing the ammonium produced after nitrite reduction by the latter species.

In contrast to the higher marine fungi, marine Phycomycetes grow poorly on inorganic nitrogen sources. Alderman and Jones (1971) demonstrated that Althornia crouchii Jones and Alderman did not use nitrate or ammonium. Thraustochytrium multirudimentale Goldstein and T. motivum Goldstein (Goldstein, 1963a) did not use KNO_3 or $(NH_4)_2SO_4$. Thraustochytrium roseum (Goldstein, 1963c) and Schizochytrium aggregatum Goldstein (Goldstein and Belsky, 1964) grew poorly on $(NH_4)_2SO_4$ and did not grow on KNO_3. The majority of both higher and lower marine fungi have been shown to grow best on organic sources of nitrogen.

All of the abovementioned studies were carried out either on solid media or in liquid batch culture. The nitrogen utilization experiments reported here show that continuous culture results differed qualitatively as well as quantitatively from batch culture

results. In batch culture, isolates of Z. maritimum grew significantly better on l-glutamic acid than on inorganic nitrogen sources. However, in continuous culture there was no preference shown by either isolate for the organic nitrogen source.

The poorer growth on the inorganic nitrogen sources in batch culture may be explained by the presence of growth limiting factors in the nutrient solution. It was demonstrated in Chapter I that the pH drop which accompanies growth on ammonium can limit growth. When ammonium was substituted by nitrate or nitrite, the pronounced fall in pH did not occur. Growth was nonetheless lower on inorganic than organic nitrogen sources, possibly due to the exhaustion of phosphate, which is necessary for nitrate reductase activity (Nicholas and Scawin, 1956). It is also possible that exhaustion of molybdenum (the metallic cofactor in nitrate reductase) or some other essential microelement inhibited growth in batch culture on inorganic nitrogen sources.

Studies with low concentrations of nitrate showed that both isolates grew well in continuous culture at one tenth the nitrogen content of NSA. Thus a ten times change in the C:N ratio, from 30:1 to 300:1, did not significantly affect growth. Sguros and Simms (1963) demonstrated a requirement for a high C:N ratio in T. achrasporum, H alopallonella, O. spectabilis, and H mediosetigera. Sguros et al (1973) showed that the latter three species could grow at low nitrogen levels and at a high C:N ratio.

In the continuous culture system, both isolates grew equally on the two lowest nitrogen concentrations and on medium without nitrogen. A certain "threshold" level of nitrogen may be necessary before good growth can occur. A possible explanation could be that the very high C:N ratio (3,000:1; 30,000:1) in these experiments produced a growth inhibition effect. Growth at low concentrations of nitrate and in NSA without nitrogen was higher in continuous than in batch culture. This suggests that growth can occur in the continuous system at very low nitrogen levels, possibly on the nitrogen supplied as contaminants in reagent grade chemicals.

Recent studies (Meyers 1966; Meyers and Hoyo, 1966; Byrne and Jones, 1975 a,b) have indicated that spore production and spore germination may be as important as dry weight in measuring the physiological response of a marine fungus. Although the contents of each flask were not systematically examined for sporulation, several observations were made in the course of these experiments. There was a direct relationship between sporulation of _Z. maritimum_ FMC and lack of nitrogen in the nutrient solution. Holligan and Jennings (1972a) observed that conidiation of the marine fungus _Dendryphiella salina_ (Suth.) Pugh and Nicot was heavy at low nitrate levels. Ng _et al_ (1973) reported that conidiation of _Aspergillus niger_ Van Tiegham in continuous culture was related to the concentration of carbon and nitrogen in the culture medium. They stated that the most generally essential condition for conidiation was the absence or near absence of

available nitrogen in the presence of assimilable carbon. Zalerion maritimum FMC, on the other hand, sporulated heavily at low concentrations of nitrogen and in the absence of assimilable carbon.

When concentrations of carbon and nitrogen were both lowered, thus keeping the carbon to nitrogen ratio constant, results differed between isolates and between batch and continuous systems. In batch culture lack of glucose reduced growth of both isolates when glucose concentrations were reduced tenfold. In continuous culture, good growth occurred at .1 mg N/l and a C:N ratio of 30:1. However, at 1 mg N/l and a C:N ratio of 3,000:1, growth was no higher than on nutrient solution without nitrogen. This would indicate that the C:N ratio may affect growth in continuous culture of Z. maritimum H 135.

In summary, the results indicate that both isolates of Zalerion can grow on low concentrations of nitrogen, and at high C:N ratios. However, results from the continuous culture system demonstrate that a C:N ratio of 3,000:1 may inhibit growth of isolate H 135. Sporulation was encouraged by low concentrations of nitrogen in the nutrient solution.

Both isolates of Z. maritimum grew to a certain extent on nearly all of the amino acids tested. Small growth increases may have been due to contaminants such as vitamins, which can adhere to organic reagents (Cochrane, 1958). Both isolates grew on each of the amino

acids most commonly found in the sea (Hood, 1963): threonine, serine, isoleucine, leucine, glutamic acid, aspartic acid, alanine, and valine. Growth of both isolates was particularly good on alanine, possibly because this amino acid participates in transamination reactions. Growth was also good on arginine, an amino acid related to nitrogen containing storage products in wood. The ring containing amino acids appeared to function in pigment formation in Isolate FMC.

Isolate H 135 grew well on the monoaminodicarboxylic acids and their amides, as did *Halosphaeria mediosetigera*, *Culcitalna achraspora*, and *Humicola alopallonella* (Sguros *et al*, 1973).

Zalerion maritimum grew well on most of the organic nitrogen sources other than amino acids which were tested. Urea was a good source of nitrogen for *Z. maritimum*, as it was for *H. mediosetigera*, *H. alopallonella*, and *C. achraspora* (Sguros *et al*, 1973). On the other hand, Johnson *et al* (1959) found that most of the marine species tested grew poorly on urea. The wood storage products allantoin and citrulline (Kremers, 1963) supported good growth of both isolates. This suggests that *Z. maritimum*, which grows on cellulosic substrates in the marine environment, may be able to utilize the nitrogen in wood.

To date, marine mycologists have studied the effects of nitrogen sources supplied singly in the nutrient solution. In nature, *Z. maritimum* grows in an environment containing various inorganic and

organic nitrogen sources. Thus, an attempt was made to determine what nitrogen sources were taken up when a combination of forms was supplied.

Chemical analysis of the nutrient solution demonstrated that Z. maritimum took up ammonium to the complete exclusion of nitrate. It can therefore be assumed that the presence of ammonium inhibited nitrate reduction, probably by repressing the synthesis of nitrate reductase (Morton and MacMillan, 1954; Kinsky, 1961; Cove and Pateman, 1969). Sguros and Simms (1963) suggested that H. mediosetigera, T. achrasporum, H. alopallonella, and O. spectabilis preferentially utilized ammonium to nitrate, because of the rapid pH drop in media containing ammonium. Preferential utilization of ammonium over nitrate is generally acknowledged (Morton and MacMillan, 1954; Nicholas, 1965) to be the pattern in terrestrial fungi. The usual evidence presented for this theory is a sharp drop in pH during early growth.

Analyses of the nutrient solution suggested that Z. maritimum took up nitrite at a slower rate than ammonium. There was no removal of nitrite during the first two days of growth, a lag time which may have been necessary for the induction of nitrite reductase. Morton and MacMillan (1954) showed that ammonium and nitrite were simultaneously utilized by Scopulariopsis brevicaulis.

The fact that a nutrient has been removed from the culture medium does not necessarily mean that the nutrient has been utilized. It is possible that a certain proportion of the nutrients may have been adsorbed to the cell wall. Detection inside the cell by mass spectroscopy or tracer methodology does not prove that a nutrient has been utilized. Both ammonium and amino acids form pools within the mycelium, from which they may or may not be removed for amination, transamination, or the synthesis of cellular protein. There is evidence that Zalerion requires a nitrogen source for good growth. Thus we can assume that a portion of the nitrogen which was removed from the nutrient solution was used in cellular metabolism.

Glutamic acid was taken up either at a faster rate than nitrate (Isolate H 135) or to the complete exclusion of nitrate (Isolate FMC). Amino acids are thought to be incorporated directly into protein without prior degradation to ammonia (Nicholas, 1965; Sguros et al, 1973). It is therefore logical that glutamic acid would be more readily taken up than nitrate, which must be reduced to nitrite and ammonia before protein synthesis can occur.

Both isolates simultaneously removed both amino acids and ammonium from the nutrient solution. The amino acids were not removed at equal rates. Glutamic acid and aspartic acid, each of which participates in transaminaton reactions, were taken up by Isolate FMC in small quantities. This is in apparent contradiction to the results

which show that glutamic acid was completely removed from the nutrient solution when supplied in combination with nitrate. The poor uptake of glutamic acid and aspartic acid may be explained by the presence of ammonium in the nutrient solution. Robinson, Anthony, and Drabble (1974) found that ammonium was used before glutamic acid during condial germination and growth of Aspergillus clavatus. They suggested that ammonium acted from within the cell, possibly repressing the synthesis of the permease necessary for the transport of glutamic acid. An additional explanation may be that there were inhibitory interactions in the mixture of amino acids (Nolan, 1970), or that there was competition for uptake between the amino acids (Jones, 1963). The optimal pH for the acidic amino acid transport system is approximately 5.5 (Hunter and Segel, 1971), and the neutral amino acids may have been more readily taken up at the pH range (6.0-7.5) of the nutrient solution during these experiments.

The rapid removal of the other amino acids was not related to their ability to support growth. Alanine, an excellent nitrogen source for both isolates, was removed at the same rate as isoleucine or leucine. Previous work with terrestrial fungi (Zalokar, 1961; Gottlieb, Moitoris, and Etten, 1968; Hunter and Segel, 1971) showed that amino acids are removed from the nutrient solution and stored in internal pools, from which they may or may not be withdrawn for incorporation into cell protein or other cellular reactions. The rate of uptake is not directly dependent on subsequent metabolism (Jones,

1963), and amino acids may be taken up by the cell even though they do not support growth when supplied singly as nitrogen sources (Nolan, 1970).

Peters et al (1975) studied the composition of the free amino acid pool in 10 species of higher marine fungi which had been grown on yeast extract as a nitrogen source. They found the amino acid composition of all species to be qualitatively similar, each containing alanine, glycine, valine, proline, leucine, isoleucine, serine, threonine, hydroxyproline, aspartic acid, methionine, glutamic acid, phenylalanine, ornithine, lysine, tyrosine, tryptophan, cystine, cysteine, and histidine. They speculated that marine fungi may supply hydroxyproline, a component of collagen, and other amino acids to detritus feeders in the marine environment.

The results suggest that Z. maritimum did not carry out nitrogen fixation, denitrification to atmospheric nitrogen, or nitrification. Nitrification using an organic nitrogen source was not investigated, and it is therefore possible that Zalerion could convert a substance such as peptone to ammonium, nitrite, or nitrate. These investigations showed that Zalerion did not grow anaerobically, although the low oxygen conditions characteristic of a candle jar did not inhibit growth. Considering the low concentrations of dissolved oxygen present in the "oxygen minimum" layer and marine sediments, the effect of various concentrations of dissolved oxygen on growth of marine fungi would be a worthwhile area of future investigation.

The results from *in vitro* studies do not necessarily represent the physiological response of an organism in its natural environment. Wood (1965, p 8) summarizes the problem in the following statement:

> "The study of the physiology and biochemistry of microorganisms in pure cultures is fraught with many dangers, for microorganisms, like their macro-counterparts, can be trained even by the very artificiality of their laboratory environment to perform strange tricks which they would scorn to do in their natural state."

Marine mycologists have not yet developed the techniques for *in situ* investigations of nutritional problems such as nitrogen utilization. If one attempts to grow the organism on its natural substrate, one is confronted with the problem of not knowing the chemical composition of the growth medium. It is then impossible to tell what nutrient or combination of nutrients has produced the observed growth response. It is therefore necessary to use a chemically defined nutrient solution for comparative growth studies on specific nutrients. Such studies indicate whether the fungus is able to carry out a given reaction or utilize a certain nutrient. The accuracy with which such results can be extrapolated to the marine environment depends upon how closely conditions of cultivation such as nutrient composition and nutrient concentration parallel those of the natural substrate. It also depends on whether the system under investigation is variable with respect to the conditions of cultivation. For example, cellulose degradation by *Z. maritimum* is variable and dependent upon the composition of the growth medium

(Moore and Meyers, 1962). Patterns of nitrogen nutrition, on the other hand, can be relatively stable. Morton and MacMillan (1954) found that nitrogen nutrition in Scopulariopsis brevicaulis was not affected by presence or absence of agar, type of sterilization of nutrients, and presence or absence of vitamins. They concluded that their nutritional results reflected the true physiological state of the fungus.

The use of a chemically defined nutrient solution was necessary in this study given the nutritional nature of the problem. It is hoped that the use of a relatively dilute nutrient solution, the use of a continuous flow culture system, and the use of ion uptake measurements gave a sufficiently true picture of physiological activity to allow a comparison between laboratory results and the marine environment.

Zalerion maritimum and other higher marine fungi inhabit cellulosic substrates such as wood and leaves which are submerged, continuously or intermittently, in the sea. These substrates may be found on the ocean bottom, in the intertidal zone, or as driftwood, deadheads, or pilings. Zalerion maritimum has also been isolated from marine sediments (Höhnk, 1956). Studies to date indicate that Z. maritimum has a limited vertical distribution. Jones and Le Campion-Alsumard (1970) did not isolate Z. maritimum below 126 m.

The mycelium of *Z. maritimum* penetrates the waterlogged substrate and produces reproductive structures on the surface. Thus both seawater and the substrate are available to the fungus as a source of nutrients.

The most abundant form of nitrogen in seawater is molecular nitrogen, which amounts to 20 times the amount of combined nitrogen (Vaccaro, 1965). Combined nitrogen can be present in several forms, the availability of which is dependent upon biological activity. In the open ocean, combined nitrogen is principally found as nitrate (62%), organic nitrogen (37%), and ammonium and nitrite (1%) (Vaccaro, 1965). In coastal areas, this ratio can be altered by planktonic blooms and other biological activity. In surface waters during the summer months, the ammonium concentration may exceed the combined concentrations of nitrate and nitrite. An approximate figure for the amount of nitrogen available in seawater is 450 μg/l (Martin, 1970). Concentrations of combined nitrogen increase with depth, due to biological activity at the sediment-water interface (Vaccaro, 1965).

Nitrogen in undecayed wood is primarily present as protein. Asparagine, glutamine, allantoin, allantoic acid, and citrulline (closely related to arginine) are all important in nitrogen storage (Kremers, 1963). Various proteins are found in the bark and sieve tube liquid may contain various amino acids. The nitrogen content of mature wood is low; the proportion of protein varies from as high as 20% in young tissues to less than 1% in mature wood (Kremers, 1963).

Axenic culture of Z. maritimum and other marine fungi on wood blocks indicates that previous "digestion" of wood nutrients is not a necessary precursor to fungal growth (Johnson and Sparrow, 1961). However, in the sea Z. maritimum is growing in combination with other decay-causing organisms such as Limnoria, Bankia, and marine bacteria. It is possible that these organisms could excrete nitrogen containing products which could be used by Z. maritimum and other marine fungi. According to Ray (1959) there have been no nitrogen-containing excretory products identified from Limnoria, which has no recognizable excretory organ. She suggested that Limnoria may utilize efficiently the very low nitrogen concentrations in wood.

It is possible that marine bacteria, which are numerous in and above the sediments, may produce ammonium from the decay of wood and the organisms assocated with submerged wood (i.e. diatoms, protozoans, bladed algae, Limnoria, and Bankia). Concentrations of ammonium are higher above the sediments than in the rest of the water column, due to the decompostion of organic material (Kriss, 1963).

Both isolates of Z. maritimum grew on nitrate, nitrite, ammonium, and organic nitrogen, when supplied as sole nitrogen sources. Of greater interest was the preference shown for certain of these nitrogen sources. Of all the inorganic nitrogen sources provided the most reduced form, ammonium, was preferred. Ammonium was probably used first because it can be directly incorporated into cellular

protein, wheres nitrate and nitrite must first be enzymatically converted to ammonium.

Several of the amino acids tested served as good nitrogen sources for *Z. maritimum*. Like ammonium, glutamic acid was taken up from the nutrient solution in greater quantities than nitrate. When a mixture of amino acids was supplied in combination with ammonium, both were taken up simultaneously from the nutrient solution. We can conclude that the forms of nitrogen most readily utilized by *Z. maritimum* were ammonium and organic nitrogen. Interpreting these results in terms of the habitat of *Z. maritimum* and other marine fungi, I would suggest that the fungus utilized the organic nitrogen in wood and the ammonium produced as an organic decay product. Ammonium would be the form of nitrogen most available to fungi in sediments, because of the reducing environment created by the low dissolved oxygen concentration. I would also assume that ammonium and dissolved organic nitrogen, if present in seawater in sufficient quantities, would be absorbed by the spores and mycelium on the surface of the wood.

Zalerion maritimum can grow on low concentrations of nitrogen, and at low C:N ratios. This feature may indicate an adaptation to growth on wood, as C:N ratios in heartwood may vary from 500:1 to greater than 1,250:1 (Griffin, 1972). Wood rotting terrestrial fungi such as *Polyporus versicolor* can adapt their mycelial nitrogen content when grown on media low in nitrogen (Merrill and Cowling, 1966).

Other investigators have demonstrated that marine fungi can produce compounds which may be used as food by marine animals. Kirk and Catalfomo (1970) demonstrated the presence of ergosterol and choline in _Z. maritimum_ and other marine fungi, and suggested that marine fungi may directly supply vitamins to marine nematodes. Block _et al_ (1973) showed that the mycelium of marine fungi contained high yields of fatty acids, which could be important sources of energy for filter feeding beach animals. Peters _et al_ (1975) suggested that hydroxyproline and other amino acids found in marine fungi may provide nitrogen to detritus feeding animals.

This study indicated that _Z. maritimum_ either converted or excreted nitrogenous products which could possibly be utilized by other organisms. Growth of _Z. maritimum_ on nitrate produced measureable quantities of nitrite; growth on a mixture of amino acids resulted in the excretion of two new amino acids into the nutrient solution. Growth of _Z. maritimum_ FMC on amino acids and ammonium indicated that ammonium can leave as well as enter the fungal cell.

I suggest the following hypothetical role of _Zalerion maritimum_ in the marine nitrogen cycle: an ability to utilize nitrate, nitrite, ammonium, and a variety of organic nitrogen sources, the latter of which are available either dissolved in the ocean or in the substrate; an inability to nitrify, denitrify, or fix atmospheric nitrogen; an ability to excrete or convert nitrogen containing compounds which may

possibly be used by other marine organisms, an ability to serve as direct sources of organic nitrogen for animals feeding on fungal mycelium.

SUMMARY

A continuous culture system was developed for the cultivation of marine fungi, in order to more closely approximate the marine environment than the batch culture system. Growth of H. alopallonella, M. pelagica, and Z. maritimum was higher in this apparatus than in batch culture.

Development of the continuous culture system led to an investigation of growth dynamics in batch culture. In ammonium containing medium, a pH drop in batch culture inhibited growth of all species tested. Improved pH control was obtained with MES (2-[N-Morpholino] ethanesulfonic acid). Chemical analyses of nutrients showed that phosphate but not glucose was exhausted from the nutrient solution. All of the ammonium and none of the nitrate was removed during growth.

These investigations should permit two improvements in future nutritional studies of the marine fungi. The continuous system should allow the use of a more dilute culture medium, and the nutrient uptake information should allow the formulation of a more balanced nutrient solution.

Continuous and batch culture systems were used to study nitrogen nutrition in Z. maritimum. Batch culture results differed qualitatively and quantitatively from continuous culture results. The preference shown by Z. maritimum for the organic nitrogen source in batch culture was not shown in continuous culture.

Zalerion maritimum used various inorganic and organic nitrogen sources. When nitrogen sources were added in combination, Z. maritimum preferred either the most reduced inorganic nitrogen source or the organic nitrogen source. It was concluded that Z. maritimum preferred the nitrogen sources available in wood or decaying organic material (ammonium, organic nitrogen) to the nitrogen sources most commonly available in seawater (molecular nitrogen, nitrate). The pattern of nitrogen nutrition shown by this marine fungus was similar to that shown by many terrestrial fungi.

The experiments described in this thesis indicated that Z. maritimum did not fix atmospheric nitrogen, nitrify, or denitrify inorganic compounds to nitrogen gas. Zalerion maritimum excreted nitrogenous products such as nitrite and amino acids, which could conceivably be used as nutrients by other marine organisms.

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