Fermentation and anaerobic decomposition in a hot spring microbial mat
by Karen Leigh Anderson

A thesis submitted in partial fulfillment of requirements for the degree of Master of Science in Microbiology
Montana State University

Abstract:

Fermentation was investigated in a low sulfate hot spring microbial mat (Octopus Spring) according to current models on anaerobic decomposition. The mat was studied to determine what fermentation products accumulated, where in the mat they accumulated, and what factors affected their accumulation. Mat samples were incubated under dark anaerobic conditions to measure accumulation of fermentation products. Acetate and propionate (ca. 3:1) were the major products to accumulate in a 55&deg,C mat. Other products accumulated to a much lesser extent. Incubation of mat samples of varying thickness showed that fermentation occurred in the top 4mm of the mat. This has interesting implications for fermentative organisms in the mat due to the diurnal changes in mat oxygen concentrations. Fermentation measured in mat samples collected at various temperatures (50&deg,-70°C) showed acetate and propionate to be the major accumulation products. According to the interspecies hydrogen transfer model, the hydrogen concentration in a system affects the types of fermentation products produced. At a 65° C site, with natural high hydrogen levels, and at a 55°C site, with active methanogenesis, fermentation product accumulation was compared. There was a greater ratio of reduced fermentation products to acetate, with the exception of propionate, at 65°C. Ethanol accumulated at the 65°C site, as did lactate, though to a lesser extent. Artificial induction of an elevated hydrogen environment with the addition of 2-bromoethanesulfonic acid to 55°C mat samples only produced a substantial difference in the ratio of acetate to ethanol. Mat samples incubated in the light had less acetate accumulation than corresponding samples incubated in the dark. This might be due to inhibition of product formation by photosynthetically-derived oxygen or to photoincorporation of fermentation products. A heterotrophic potential experiment showed that acetate, lactate, and ethanol had the greatest potential for uptake by the microbial population at a 65°C site. These results correlate with the lack of propionate accumulation at 65°C (propionate had the least potential for uptake at 65°C of the compounds tested), and with the accumulation of ethanol. The results also point out that placing importance on fermentation products by their accumulation data alone may be misleading.

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Karen Leigh Anderson

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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ABSTRACT

Fermentation was investigated in a low sulfate hot spring microbial mat (Octopus Spring) according to current models on anaerobic decomposition. The mat was studied to determine what fermentation products accumulated, where in the mat they accumulated, and what factors affected their accumulation. Mat samples were incubated under dark anaerobic conditions to measure accumulation of fermentation products. Acetate and propionate (ca. 3:1) were the major products to accumulate in a 55°C mat. Other products accumulated to a much lesser extent. Incubation of mat samples of varying thickness showed that fermentation occurred in the top 4 mm of the mat. This has interesting implications for fermentative organisms in the mat due to the diurnal changes in mat oxygen concentrations. Fermentation measured in mat samples collected at various temperatures (50°-70°C) showed acetate and propionate to be the major accumulation products. According to the interspecies hydrogen transfer model, the hydrogen concentration in a system affects the types of fermentation products produced. At a 65°C site, with natural high hydrogen levels, and at a 55°C site, with active methanogenesis, fermentation product accumulation was compared. There was a greater ratio of reduced fermentation products to acetate, with the exception of propionate, at 65°C. Ethanol accumulated at the 65°C site, as did lactate, though to a lesser extent. Artificial induction of an elevated hydrogen environment with the addition of 2-bromoethanesulfonic acid to 55°C mat samples only produced a substantial difference in the ratio of acetate to ethanol. Mat samples incubated in the light had less acetate accumulation than corresponding samples incubated in the dark. This might be due to inhibition of product formation by photosynthetically-derived oxygen or to photoincorporation of fermentation products. A heterotrophic potential experiment showed that acetate, lactate, and ethanol had the greatest potential for uptake by the microbial population at a 65°C site. These results correlate with the lack of propionate accumulation at 65°C (propionate had the least potential for uptake at 65°C of the compounds tested), and with the accumulation of ethanol. The results also point out that placing importance on fermentation products by their accumulation data alone may be misleading.

INTRODUCTION

This research represents a continuing effort to characterize the microbial communities in microbial mats found in alkaline siliceous hot springs. Many of these systems are located in Yellowstone National Park and have been well described by Brock (9).

My objective was to further study fermentation as a part of anaerobic decomposition in the Octopus Spring microbial mat. This system is located in the White Creek area of the Lower Geyser Basin in Yellowstone National Park. Its 91°C source supplies a continuous flow of alkaline water (pH 8.3) to the microbial mats which thrive from 74° to 30°C (51).

Rationale for Study of Hot Spring Microbial Mats

Much attention has been focused on hot spring microbial mats largely because of interest in Precambrian stromatolites, mat-like structures apparently formed by ancient microbial life (49). These fossils are comprised of laminated sedimentary rocks that house microscopic structures often morphologically similiar to filamentous microorganisms (2). Microbial mats of hot springs contain conical or columnar structures (16, 50) similar to structures found in stromatolites, and are also laminated with depth. Both the conical structures and the mats contain filamentous organisms, such as the photosynthetic bacterium, Chloroflexus aurantiacus. It is hoped that studies of modern ecosystems, such as the mats of hot springs, will reveal information relevant to these ancient structures and contribute to our understanding of early life on Earth.

Ecologically, the Octopus Spring microbial community should be a simple system for study. High temperatures restrict the diversity of microorganisms in an ecosystem (9). This is true for the mat-forming phototrophs in Octopus Spring, and is presumed valid for other microorganisms in the mats (52). Eucaryotic organisms, including metazoan grazers, are absent above 50°C (56). The thickness of the Octopus mat and the chemical and physical parameters of the source water have remained constant for many years (8, 9). The major advantage in studying the Octopus mats is the ability to investigate a natural ecosystem that remains stable with time.

Finally, there is an increasing interest in using anaerobic microbes for processing wastes from municipalities, agriculture, and industry to yield chemical and fuel products (75). Thermophilic bacteria are being used for the industrial production of fermentation products such as methane and ethanol (57, 75). A better understanding of anaerobic processes in thermophilic environments could benefit developments in industry.

Microbiology of Hot Spring Microbial Mats

Because much is known about Octopus Spring, I will consider its microbiology in detail. Few microorganisms have been identified from these mats. The cyanobacterium, Synechococcus lividus, comprises the top green layer of the mat and is responsible for the community's primary production (9, 40). Chloroflexus aurantiacus, a filamentous bacterium, makes up much of the orange undermat and is responsible for much of the mat's integrity (17). Little is known about the aerobic organisms in this system. The aerobic bacterium, Thermus aquaticus (10, 52) was isolated from Octopus Spring. Microscopy indicated that Isocystis pallida, a filamentous chemoheterotrophic bacterium, is also an inhabitant of Octopus Spring (Ward, personal communication, 19). Workers have primarily focused on anaerobic processes in this system because it was presumed that the aerobic zone, produced by the top layer phototrophs (17), was thin relative to the thickness of the mat. This, together with other reasons to be discussed below, and the interest in the economic potential of thermophilic anaerobes explains why the majority of organisms cultured were anaerobic and fermentative. A sulfate-reducer, Thermodesulfobacterium commune (77) and a methanogenic bacterium, Methanobacterium thermoautotrophicum (76) have also been isolated. The fermentative bacteria will be considered in greater detail below.

How these organisms interact has been the subject of previous research. Doemel and Brock (17) suggested that the mat was divided into two major zones: an upper, aerobic zone in which adequate light is available for photosynthesis, and a lower, dark anaerobic zone where decomposition predominates. A number of studies confirm a photosynthetically-active upper mat. Light does not penetrate below 2 mm from the mat's surface due to shading by Synechococcus and the primary photic zone is restricted to the upper 0.5 to 1 mm (17). The highest concentration of chlorophyll a, representing Synechococcus, is found within the top 0.5 mm and is absent below 1 mm. The highest levels of bacteriochlorophylls a and c, presumably from Chloroflexus, correspond to the 0.5 to 3 mm interval (5). During full sunlight, oxygenic photosynthesis occurs in the 0.5 to 1.1 mm of a 55°C Octopus mat. The concentration of oxygen in the top 3 mm is about 6 times that of the overlying water and the highest level of oxygen peaked in the upper 1 mm of the mat. These observations support active photosynthesis by Synechococcus. Below 3 mm, oxic conditions are lower than the levels of oxygen in the overlying water, and anoxic conditions prevail near 7mm and below (40).

Interest in the steady-state nature of this system led to studies on microbial decomposition in the mats for a number of reasons. These mats are above the upper temperature limit of metazoan grazers (56, 61) and no fungal decomposers have been isolated (9) Consequently, the mats depend on a procaryotic food chain for the mineralization of organic matter. Doemel and Brock (17) investigated the possible steady state nature of the mat, and discovered that the growth rate equalled the decomposition rate, with complete decomposition occurring in one year. The rates of growth and decomposition tested at sites between 70° and 42°C were optimum between 55° and 52°C.

Because high temperature limits the solubility of oxygen, decomposition was thought to occur through anaerobic processes. Revsbech and Ward (40) found a diurnal change in oxygen concentrations in this system. The mats are oxic during the day, as described above, but at night they are anoxic with the exception of the top 0.5 mm. The variability of oxygen levels in this system raises the question of whether aerobic or anaerobic decomposition, or both, is important. As indicated, little is known about aerobic organisms in the mats, with much more information known about anaerobic isolates and processes. Studies on anaerobic decomposition in Octopus Spring have been formulated according to current models of this process. Before detailing these studies, it is important to review proposed models for anaerobic decomposition.

Current Models of Anaerobic Decomposition

Presently investigators advocate a three-stage scheme to describe the fate of organic molecules in anaerobic systems (11, 33, 34). This scheme has been developed from observations of fermentation in a number of anaerobic environments, including sewage sludge and other waste digesters, sediments of lakes, rivers, and marine systems, flooded soils, and sediments from the tundra, swamps, and bogs (11). Other systems studied include the rumen of herbivores and the caecum of certain non-ruminants as well as the gastrointestinal tract of humans and animals (33).

The stages of decomposition are divided by the type of microorganism involved in the process. Fermentative bacteria are responsible for degrading the carbohydrates, proteins, and lipids to fatty acids, alcohols, carbon dioxide, hydrogen, ammonia, and sulfide. The second group, obligate proton-reducing acetogenic bacteria, degrade the fermentation products propionate and longer chained fatty acids, alcohols, and possibly some organic acids, such as benzoate (18). The acetogenic bacteria convert these substrates to acetate, hydrogen, and in the case of odd numbered carbon energy sources, carbon dioxide. Finally, in low sulfate environments, methanogenic bacteria are responsible for the terminal decomposition process of methane production. Other terminal decomposition processes will be considered below. Methanogenic bacteria generate methane from acetate, carbon dioxide and hydrogen, or other substrates such as formate and methanol.

It is important to consider not only the individual processing of organic compounds by these groups, but also the interaction among them. As noted above, fermentative bacteria produce compounds which are used by the other two groups of organisms. Before the discovery of obligate proton-reducing acetogenic bacteria, it was thought that methanogenic bacteria degraded fermentation products to produce methane. This idea was dispelled, however, when Bryant discovered that an abundant sewage methanogen, Methanobacillus omelianskii, which degraded ethanol to methane, was actually a coculture of two organisms (13). The nonmethanogen, the so-called S organism, degraded ethanol to acetate and hydrogen (39), and the methanogen, designated M.o.H. and later named Methanobacterium bryantii, used the hydrogen produced in the fermentation for methane production (72).

During characterization of the S organism, researchers noted that the organism grew poorly on alcohols with little hydrogen production. When it was grown with a methanogen, its growth increased dramatically, neither ethanol nor hydrogen was produced, and methane accumulated (39). It was assumed that hydrogen production by the S organism increased in the presence of the methanogen. The increased production of hydrogen was reflected in the amount of methane produced: 4 moles of hydrogen are used to produce 1 mole of methane. The observation of increased methanogenesis in the presence of a fermentative organism was also observed by Scheifinger, et al. (44) in the growth of Selenomonas ruminantium with methane-producing bacteria. Noting the shift in fermentation products, as well as the increased production of hydrogen in coculture, researchers began investigating other culture systems.

Studies on the interaction of bacterial isolates from the rumen showed different patterns of fermentation product accumulation in pure culture versus coculture (71). The cellulytic Ruminococcus albus produced ethanol, acetate, formate, hydrogen, and carbon dioxide from cellubiose when grown alone. Vibrio succinogenes coupled the oxidation of hydrogen or formate with reduction to succinate in pure culture. When these two organisms were grown together ethanol was not produced but succinate accumulated and a rise in acetate concentration nearly equalled the amount of ethanol produced by the monoculture (22). This example, as well as many others (14, 15, 27, 28, 55, 66, 67, 70) suggested that a hydrogen-using organism or a hydrogenotroph (68) caused a shift in electrons away from more reduced fermentation products, such as ethanol, to yield more oxidized products, such as acetate.

It was also discovered that the accumulation of hydrogen inhibited the growth of some organisms. The S organism grew poorly alone when grown on ethanol but fermented ethanol to acetate and carbon dioxide in the presence of a methanogen. <u>Desulfovibrio</u> species fermented ethanol or lactate to the same products in the presence of a methanogen but grew poorly by themselves (12, 72). Propionate- and butyrate-degrading bacteria isolated from sewage sludge and aquatic sediments will not grow unless they are cultured with a hydrogenotroph (7,35). These organisms provide the first evidence of nonmethanogenic bacteria that anaerobically degrade fatty acids without light, sulfate, nitrate or similar electron acceptors (35). The butyrate-degrading organism also metabolized caproate and caprylate to acetate and hydrogen and valerate and heptanoate to acetate, propionate, and hydrogen (36). These bacteria have been termed obligate proton-reducing acetogenic bacteria, because they must produce hydrogen to grow, but require an environment in which hydrogen is removed (33, 34). The degradation reactions for the propionate- and butyrate-acetogens, for example, become thermodynamically favorable when the hydrogen stress is relieved. This is evident when comparing the free energies of proposed reactions involved in the catabolism of propionate and butyrate alone:

	$\Delta G^{o\prime}$(kcal/rxn)
propionate + $3H_2O \rightarrow$ acetate + HCO_3^- + H^+ + $3H_2O$	18.2
butyrate + $2H_2O \rightarrow 2$acetate + H^+ + $2H_2$	11.5

to the reduction in free energies to -24.4 and -9.4 kcal per reaction, respectively, when grown in syntrophic association with hydrogen-using methanogens (11).

The importance of hydrogen regulation in an ecosystem is explained in a concept known as interspecies hydrogen transfer (33, 34). In glycolysis, the regeneration of NAD^+ by fermentative bacteria is accomplished by shifting electrons from NADH towards the production of various reduced products, such as ethanol, lactate, formate, propionate, or hydrogen (74). The concentration of hydrogen in the system determines whether reduced fermentation products or hydrogen will be formed. This can be seen in the following thermodynamic reaction:

$$NADH + H^+ \rightarrow H_2 + NAD^+ \qquad \Delta G^{o\prime} = +4.3 \text{ kcal/rxn}$$

Note that this reaction is endergonic, and thus oxidation of NADH to produce hydrogen will not be feasible until the products, namely hydrogen and NAD$^+$ are removed from the system (34). Hydrogen removal by hydrogenotrophs, such as methanogens, will force the reaction to the right and allow fermentative organisms to produce more oxidized products (33). Not only does interspecies hydrogen transfer result in a different proportion of reduced fermentation products than if the fermentative organism is grown in pure culture, but an increase in substrate use was shown in several coculture experiments in which a hydrogenotroph was employed (74). More ATP is synthesized by the nonmethanogen because pyruvate can be oxidized to acetate and carbon dioxide via acetyl-CoA with the generation of 1 mole of ATP/mole of acetate formed (28, 74) and greater growth is seen for the fermentor (15, 67 ,74).

From the above observations, interspecies hydrogen transfer can be divided into two categories. The first involves nonobligatory interactions between methanogenic bacteria or other hydrogenotrophic bacteria such as sulfate-reducing bacteria, and fermentative bacteria in which hydrogen use benefits both organisms. If the hydrogenotrophic organism is removed from the coculture containing a fermentative bacterium, the fermentor finds alternative routes to dispose of its electrons--namely in shifting fermentation production towards more reduced products (71). The second category involves an interaction in which the removal of hydrogen is essential to the functioning of obligate proton-reducing acetogenic bacteria (74).

Methanogens perform two principal functions in mixed culture fermentations. Through utilization of hydrogen for methanogenesis, they keep electron flow from fermentative bacteria towards proton reduction. Thus a shift in reduced to oxidized fermentation products takes place. Acetogens are also supported by this proton transfer. And, finally, some methanogenic bacteria produce methane from acetate generated by fermentation and interspecies hydrogen transfer reactions (33).

Workers recently investigated a bacterial mixture capable of degrading sucrose to methane and carbon dioxide (23). The organisms included a fermentor, two acetogens, and two methanogens whose pure culture characteristics were known. By culturing the fermentor with various combinations of the other organisms, workers demonstrated the importance of interspecies hydrogen transfer and acetogenic action on the the types of fermentation products formed.

Interspecies hydrogen transfer has also been studied in natural systems. The ruminant system, for example, has been well characterized (21, 69, 71). Active methanogenesis in this environment maintains a low hydrogen concentration (10^{-4} atmospheres) and the dominant fermentation products are acetate, propionate, and butyrate. Ethanol and other reduced fermentation products are considered absent in the rumen due to interspecies hydrogen transfer of electrons from hydrogen to methane production (69). Obligate proton-reducing acetogens have not been isolated from the rumen. Products such as propionate and butyrate are absorbed by the rumen walls for use by the ruminant (69) and acetogenic bacteria probably would not occur to any significant extent in the rumen unless there was a reduction in the turnover of rumen contents (37).

Anaerobic decomposition has also been studied in aquatic sediments. Fermentation leading to methane production as the the terminal decomposition process was noted in several systems (29, 54, 62, 64, 65) and acetate is the primary substrate for methanogenesis in aquatic sediments (11). Studies on microbial populations in sediments from a eutrophic lake indicated the importance of interspecies hydrogen transfer (25). Sediments labeled with [U-^{14}C] glucose and incubated with 100% hydrogen produced less acetate and more lactate than corresponding samples incubated with 100% nitrogen. Evidence for the presence of acetogenic bacteria in an aquatic system was noted in a freshwater river bed (4) and in a eutrophic lake sediment (29). In the river sediment, butyrate turnover was inhibited by the addition of hydrogen. In the lake sediment, hydrogen completely inhibited the metabolism of propionate, iso-butyrate, iso-valerate, and valerate added to the sediment, whereas greater than 90% of added volatile fatty acids were metabolized in controls. Inhibition of methanogenesis in these sediments also resulted in an immediate accumulation of hydrogen and fatty acids.

The importance of methane-producing bacteria in a decomposition scheme is fully realized when reviewing the effects of a high hydrogen environment on an anaerobic community. These are the most important organisms capable of catabolizing acetate and hydrogen to gaseous products in the absence of light energy or exogenous electron acceptors such as oxygen, sulfate, and nitrate. Without the methanogenic bacteria, effective mineralization would stop because nongaseous reduced products of fermentation would accumulate (11).

Sulfate-reducing bacteria can outcompete methanogens for available hydrogen and acetate in high sulfate environments (11, 54) and should be considered when studying anaerobic decomposition. Marine environments (3, 26, 45, 47) and a hot spring microbial mat (53) are examples of high sulfate environments with active sulfate reduction. Addition of sulfate to low sulfate environments was also shown to inhibit methanogenesis with a concomitant stimulation of sulfate reduction (63). The major acetate-users in a saltmarsh and in marine sediments were sulfate-reducing bacteria (3, 47). The oxidation of short-chain fatty acids was noted associated with sulfate reduction (26). The addition of sodium molybdate, an inhibitor of sulfate reduction, stopped propionate and butyrate degradation (3, 47) as well as the mineralization of propionate, lactate and free amino acids (46).

These observations suggested that sulfate-reducing bacteria are important in anaerobic decomposition. A two stage process has been proposed for the fate of organic compounds in high sulfate environments. Fermentative bacteria perform the first stage of degradation and sulfate-reducing bacteria oxidize reduced fermentation products, thus fulfilling the roles of acetogens and methanogens in low sulfate systems (54).

Anaerobic Decomposition in Hot Spring Microbial Mats

Investigations of anaerobic decomposition in hot spring microbial mats suggest that both the 2 and 3 stage models exist. Ward and Olson (53) showed that sulfate reduction dominated methanogenesis in Bath Lake, a high sulfate hot spring. Acetate and propionate, as well as other volatile fatty acids, accumulated as sulfate was depleted in samples incubated under dark anaerobic conditions. These observations suggest a 2 stage model for anaerobic decomposition in Bath Lake. Previous work on the Octopus Spring microbial mat suggests a 3 stage model for anaerobic decomposition. In this low sulfate environment, methanogenesis is active (51) and hydrogen and carbon dioxide, not acetate, are important methane precursors (43). Radiolabeled acetate added to the mats was incorporated by long filamentous organisms resembling the phototrophic bacterium from mat communities, *Chloroflexus aurantiacus* (43). Tayne (48) investigated the fate of acetate together with other fermentation products and found that acetate, propionate, butyrate, lactate, and ethanol were photoincorporated by a strain of *Chloroflexus* isolated from the mat. Catabolism of these compounds in the dark, especially under dark anaerobic conditions, was not significant, with the exception of lactate, which was catabolized under all incubation conditions. Tayne also found evidence for butyrate acetogenesis, although this was not considered an important process in the mats (48). Recycling of fermentation products to *Chloroflexus* was proposed as an alternative to fermentation product catabolism often noted in other natural systems.

Little is known about fermentation in Octopus Spring. Doemel and Brock (17) showed that the concentration of protein decreased with depth in the mat, implicating fermentation processes. As mentioned above, more fermentative bacteria have been isolated from Octopus Spring than any other metabolic group (52). Table 1 lists these saccharlytic organisms and their characteristic fermentation substrates and products.

Table 1. Fermentation organisms isolated from Octopus Spring and their known fermentation substrates and products.

ORGANISM	KNOWN SUBSTRATES	FERMENTATION PRODUCTS
THERMOANAEROBIUM BROCKII (76,78)	SUGARS, CARBOHYDRATES	ETHANOL, LACTATE, ACETATE, CO_2, H_2
THERMOBACTEROIDES ACETOETHYLICUS (6)	SUGARS, CARBOHYDRATES	ETHANOL, ACETATE, H_2, CO_2
THERMOANAEROBACTER ETHANOLICUS (59)	SUGARS, PYRUVATE	ETHANOL, CO_2, ACETATE, LACTATE H_2
CLOSTRIDIUM THERMOHYDROSULFURICUM (60,76)	SUGARS, CARBOHYDRATES, PYRUVATE	ETHANOL, LACTATE, ACETATE, CO_2, H_2
CLOSTRIDIUM THERMOAUTOTROPHICUM (58)	SUGARS	ACETATE

My role in the continuing investigation of anaerobic decomposition in the Octopus Spring microbial mats has been to investigate fermentation. The following aspects of fermentation were addressed:

1) What fermentation products accumulate?
2) Where do these products accumulate in the mat?
3) What factors could affect the accumulation of these products?

The studies designed to answer these questions attempted to further investigate a unique system in which a photoheterotroph appears to play a role in the fate of fermentation products.

MATERIAL AND METHODS

Study Area

Experiments were carried out at Octopus Spring located in the Lower Geyser Basin of Yellowstone National Park (see 17 for specific location). This area was chosen to study decomposition as it has been the subject of previous investigations on anaerobic processes (17, 51, 52, 53) and the microbial system has been well studied by others (5, 9). Much of the work was performed at a site south of the main source. This shoulder area was separated from the source by a sinter barrier which allowed a gentle flow of water over the microbial mat. Samples were more homogeneous and temperature fluctuations were less (55°C ± 2°C) in the shoulder than at other areas of the Spring. Samples were also collected from sites at 50°, 60°, 65°, and 70°C in the southern effluent channel.

Another study area, Mushroom Spring, also located in the Lower Geyser Basin (9) has a microbial mat similar in structure and composition to that found at Octopus Spring. The influence of light on fermentation product accumulation was studied at a 55°C Mushroom Spring site.

Accumulation of Fermentation Products

Unless otherwise noted, all experiments designed to measure the accumulation of fermentation products produced under dark anaerobic conditions were performed as follows. Vertical core samples removed from the microbial mat with a #4 cork borer (1 cm x 50.3 mm^2) were placed in 1 dram glass vials (14.5 x 45 mm, Kimble). Anaerobic conditions were established by continuously flushing a stream of nitrogen gas over the contained samples. Vials were sealed with butyl rubber stoppers (00, Thomas) and then wrapped at the glass-rubber interface with black electrical tape to secure the stoppers during incubation. Dark conditions were simulated by wrapping the vials with black electrical tape and several layers of aluminum foil. Usually 1 ml of source water, which had been bubbled with either helium or nitrogen, was added to each core sample. Vials were incubated at in situ temperatures during the collection procedure. For transport to laboratory incubators, vials were transferred to plastic thermos bottles containing water at the in situ temperature and the bottles were placed in styrofoam coolers containing water 5-10°C warmer. Transportation time to the laboratory was 2-3 hours, during which time the temperature in the incubators fell 5-8°C. Vials were incubated in darkened incubators that maintained a set temperature to within ±5°C.

During dark anaerobic incubations in the lab, subsamples were removed from the gas headspace and measured directly by gas chromatography. Liquid was subsampled and frozen (-20°C) for later analysis of fermentation products.

Depth Profile

From a 55°C site 1 cm cores were sectioned with a razor blade into the following vertical intervals: the top 1 mm, 0-2 mm, 0-4 mm, 0-6 mm, 0-8 mm, and 0-10 mm. These were treated as specified above for studies on fermentation product accumulation.

Temperature Distribution

One centimeter cores were sampled from the shoulder at 50° and 55°C and from the southern effluent channel at 60°, 65°, and 70°C. Vials containing samples from each temperature were injected with 2 ml of anoxic source water and transported to the laboratory as described above. Upon returning to Montana State University, it was noted that the temperature of each transporting thermos had equilibrated to 65°C. The incubations, however, were continued in laboratory incubators at 50°, 55°, 60°, 65°, and 70°C and subsamples were removed at time intervals for fermentation product analysis.

Factors Affecting Fermentation Product Accumulation

Inhibition of Methanogenesis

Tayne (48) showed that methane production in cores incubating under dark anaerobic conditions could be inhibited by the addition of 2-bromoethanesulfonic acid. A concomitant rise in hydrogen accumulation was noted with the inhibition of methanogenesis.

A #6 cork borer (1 cm x 78.5 mm^2) was used in these experiments as larger liquid volumes were needed or both volatile and nonvolatile fatty acid analysis. Cores sampled at 55°C and in the southern channel at 65°C were incubated using 2 dram glass vials (19 x 48 mm, Kimble), butyl rubber stoppers (01, Thomas), and 3 ml of anoxic source water. Samples were monitored for methanogenesis. When methane levels reached approximately 1.0 µmole/vial (48), 0.2 ml of a 0.5 M stock solution of 2-bromoethanesulfonic acid (Sigma Chemical Company), adjusted to pH 6.4 to match the pH of incubating cores, was injected into half of the vials to obtain a final concentration of 0.05 M; the other half of the samples served as controls. Both gas and liquid subsamples were removed at intervals until the completion of the experiment.

Light

Cores removed from a 55°C Octopus Spring site were incubated with 1.5 ml of anoxic source water. Vials were flushed with either nitrogen or helium and incubated glass end up in situ under light or dark conditions for 5 and 10 hours. At the end of each incubation, samples were injected with 0.15 ml of a 37% formaldehyde solution (formalin) and shaken to stop all biological activity. All formalin killed samples were assayed for fermentation products. Light intensity was monitored with a Li-Cor light meter (model LI-185).

This experiment was repeated twice in Mushroom Spring at a 55°C site. After 5 1/2 and 7 hours of incubation for each experiment, respectively, 1 ml syringes were used to transfer liquid subsamples from vials to 1.5 ml plastic centrifuge tubes (Thomas). These subsamples, designated for later fermentation product analysis, were kept cool on ice in a styrofoam incubator during transport to the lab.

Heterotrophic Potential

Photoheterotrophy was investigated in a bioassay of adaptation by the microbial mat's population to take up specific compounds. The method of Hobbie and Wright (73) was used to determine a 65°C population's potential for assimilating radiolabeled fermentation products. T/F values (where T is the incubation time in hours and F is the dpm of labeled cells and CO_2 divided by the total dpm added per vial) were regressed on A (the concentration of added substrate in μM). The reciprocal of the slopes of the regression lines gave the V_{max} values for each compound tested. The V_{max} values represented the microbial population's potential to take up the compounds tested.

The method for measuring heterotrophic potential for a 65°C microbial population closely followed that of Tayne (48). Thirteen 1 cm cores gathered with a #4 cork borer were sectioned to obtain the top 1-3 mm interval. This section was used as it gave a maximum for uptake and metabolism of fermentation products compared to other intervals tested in a 50°C Octopus Spring mat (48). The subsamples were homogenized in a 40 ml hand tissue grinder (Wheaton) and diluted in 150 ml of Octopus Spring source water. Two milliliters of the homogenate was added to 1 dram glass vials. These were sealed with butyl rubber stoppers and the closures were secured with black electrical tape. Vials placed glass side up in a wire rack in the flowing water were preincubated for 30 minutes under full sunlight. Ten-fold concentrated stock solutions of 1-^{14}C labeled acetate, propionate, butyrate, lactate, and ethanol were prewarmed in the Spring. After preincubation, each vial was injected with a radiolabeled fermentation product and returned to the immersed wire rack. The final concentrations obtained of labeled compounds were approximately 0.125, 0.250, 0.5, and 1.0 µCi/vial, with the exception of ethanol and acetate, which were three times higher (see Analytical Methods). Vials were injected with 0.1 ml of formalin after incubation for 30 minutes.

For each radiolabeled compound tested, triplicate samples at each of the four concentrations were analyzed for $^{14}CO_2$ in the vial headspace, and for the presence of the radiolabel in the cell fraction and filtrate from the homogenate.

Analytical Methods

Gas Analysis

Hydrogen and methane were measured by removing 0.2 ml subsamples from vial headspaces with a 1 ml Glaspak syringe (Becton, Dickinson) modified with a Mininert valve (Supelco) to make it gas-tight. Subsamples were injected into a gas chromatograph (Carle, model 8500) equipped with a thermal conductivity detector and stainless steel column (2.3 meters by 3.18 mm O.D) packed with Poropak N

(80 mesh). The oven temperature was set at 42°C. Nitrogen was used as a carrier gas with a flow rate of 21 ml/min. The column had been standardized with known concentrations of hydrogen and methane, and the area unit responses of samples injected were corrected to µmoles/injection by an integrating computer (Spectra Physics model 4100). Total hydrogen and methane per vial were calculated by correcting for headspace volume.

Fermentation Product Analysis

Liquid subsamples (0.2 ml) removed with a 1 ml syringe flushed with nitrogen were placed in 1.5 ml plastic centrifuge tubes and frozen (-20°C) until analysis. Preparation of these samples for volatile fatty acid and alcohol analysis (Rutherford, personal communication) was as follows. Thawed subsamples were acidified with 80 µl of a 40% (w/v) aluminum sulfate solution and 4 µl of a 40 mM hexanoic acid solution was added as an internal standard. Vortexed samples were filtered through 0.45 µm membrane filters (13 mm, Millipore type HA) contained in Swinnex filter holders. Two microliters of the filtrate was injected into a temperature-programmable gas chromatograph (Varian model 3700) fitted with a glass column (6 feet x 0.25 inches O.D., 2 mm I. D.) packed with GP 15% SP-1220/1% H_3PO_4 on Chromosorb W, AW (Supelco, mesh size 100/120). The injector and flame ionization detector were set at 170°C and 250°C respectively. During an analysis the oven temperature was programmed to hold 105°C for 2 minutes, increase at a rate of 40°C/min to 145°C, and hold this final temperature for 3 minutes. Flow rates for the detector gases were 300 ml/min for air and 30 ml/min for hydrogen and the carrier gas, helium, was set at 30 ml/min. Two standard solutions, a volatile fatty acid (VFA) rumen standard and an alcohol standard mixture, (both from Supelco), were used to calibrate area unit responses (µM) monitored by the integrating computer. The standard solutions could not be mixed to calibrate the chromatograph, as similar retention times for acetate from the VFA solution and pentanol from the alcohol solution made it impossible to accurately calibrate the two compounds. Each solution was, therefore, treated individually with aluminum sulfate and hexanoic acid, as described above, for calibration. A program was established on the integrating computer which included data from both standardizations.

Twenty microliters of an internal standard, 10 mM glutaric acid, was added to subsamples (0.2 ml) which were then methylated, according to (20) for nonvolatile fatty acid (nVFA) analysis. Chromatograph settings were the same as above, except the oven temperature was initially set at 85°C for 2 minutes, then programmed to increase at a rate of 10°C/min to 145°C, which was maintained for 5 minutes. An nVFA standard (Supelco) was used to calibrate this column using an internal standard method. After each run, it was necessary to manually reset the oven temperature to 185°C to flush out a contaminating peak having a later retention time than the nonvolatile fatty acids. This higher temperature was maintained for 5 minutes before the column was cooled for another injection.

Because several liquid subsamples were sequentially removed from a vial during an accumulation study, it was necessary to adjust each chromatographic measurement with a correction factor to account for the previous amount of product removed. The mM concentration measured was adjusted to µmoles/vial by multiplying it by the liquid volume in the vial at the time of subsampling. This same mM concentration was also multiplied by the volume of subsample removed (0.2ml + 0.05ml for syringe deadspace = 0.25 ml) to determine the amount removed for analysis. Each subsequent time point was corrected first to a per tube amount and then for the amount removed in previous analysis.

After data were corrected, means, standard deviations, and standard errors were calculated for replicate samples.

Preparation of Radiolabeled Compounds

The ^{14}C-labeled fermentation compounds used in the 65°C heterotrophic potential experiment were: [2-^{14}C] acetic acid (New England Nuclear, NEN), 1.8 mCi/mmol; [1-^{14}C] propionic acid (NEN), 58.4 mCi/mmol; [1-^{14}C]butyric acid (Amersham), 56 mCi/mmol; DL-[1-^{14}C]lactic acid (Amersham), 54 mCi/mmol, and [1-^{14}C]ethanol (NEN), 7.6 mCi/mmol. These solutions were diluted with anoxic filtered Octopus Spring water to obtain final concentrations of 0.125, 0.25, 0.5, and 1.0 μCi/vial and autoclaved. Total radioactive counts determined for these solutions showed that the counts for ethanol and acetate were 2 and 3 times higher, respectively. This can only be accounted for by dilution error in the original preparation of these solutions.

$^{14}CO_2$ Analysis

Subsamples from vial headspaces were injected into the thermal conductivity gas chromatograph (described above) connected by a teflon line to a gas proportional counter (Packard, model 894) for $^{14}CO_2$ analysis. The gas chromatograph had a flow rate of helium of 21 ml/min.

The gas proportional counter had two combustion furnaces in series operated at 750°C. Helium make-up gas was added after combustion to increase the flow rate to 70 ml/min. Propane, the quench gas, was added at 10% of the total flow rate. A Minigrator (Spectra Physics) recorded the gas proportional counter output.

Radiolabeled Cells and Filtrates

After $^{14}CO_2$ analysis, vials were vortexed, and a 1:10 dilution of the homogenate (0.2 ml sample, 1.8 ml of distilled water) was filtered through a Millipore filter (0.45 µm x 25 µm, type HA). With a vacuum applied to the filter apparatus, the filtrate was collected in a small glass vial suspended beneath the funnel. Air dried filters were exposed overnight to hydrochloric acid (12 N) fumes to remove carbonates. The filters were placed in 10 ml of Aquasol (NEN), and the filtrates (500 µl) plus distilled water (500µ l) were pipetted into 2 ml of Aquasol. Both were counted by a liquid scintillation counter (Packard, 460 c) using the sample channels ratio method to correct for quenching. Counter windows were set at (A) 0-156 KeVolts and (B) 4-156 KeVolts. The data were reported as dpm per sample vial (average of triplicate vials) after correction for subsample volume and dilution. Total dpm recovered as cells and filtrate was 83.99 ± 1.27 standard error for a sample size of 60 vials.

Statistical Analysis

Linear regression was used in the heterotrophic potential study to determine the slope and correlation coefficient, r, for each line plotted. A two sample Student's t test was used to compare means calculated for the light studies. Statistics programs were from Lund (31).

RESULTS

Fermentation Product Accumulation

The accumulation of fermentation products in the Octopus Spring mat at 55°C incubated under dark anaerobic conditions is shown in Figure 1. The predominant fermentation products found were the volatile fatty acids (VFA) acetate and propionate. Acetate, the major product, accumulated in a ratio of 3:1 relative to propionate. Iso-butyrate, n-butyrate, iso-valerate, and n-valerate also accumulated over time. These products, however, reached much lower concentrations than did acetate and propionate (Figure 1). The trend of fermentation product accumulation was repeated in a number of similar experiments (data not shown). There was no evidence that nonvolatile fatty acids or alcohols accumulated at 55°C during an incubation period of 120 hours (data not shown).

Location of Product Accumulation

Depth Profile

The vertical position in which fermentation occurs in the mat was investigated by studying core sections cut to vary in thickness from the top to the full length

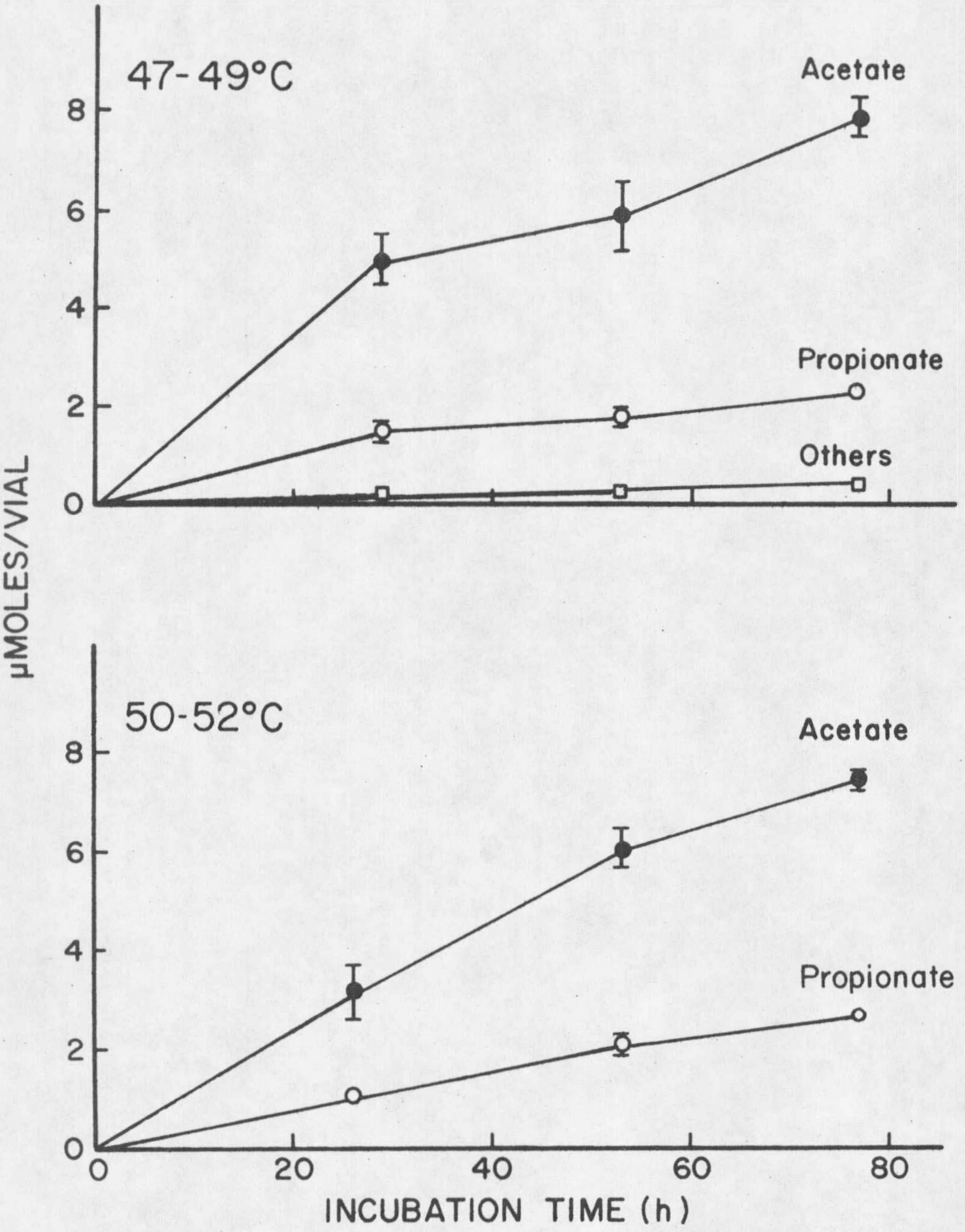

Figure 1. Accumulation of volatile fatty acids during dark anaerobic incubation of mat samples from the shoulder, 47°-49°C, and the southern effluent channel, 50°-52°C, of Octopus Spring. "Others" refers to iso-butyrate, n-butyrate, iso-valerate, and n-valerate. Bars are standard error (n=3).

of the core. Acetate and propionate were again found to be the predominant fermentation products (Figure 2). Although these fatty acids accumulated in the top green layer, their accumulation was more rapid if the thickness of the mat was increased to 2 or 4 mm. Further increases in thickness did not result in more rapid acetate and propionate accumulation.

Temperature Distribution

The cyanobacterial mats at Octopus Spring extend from sites located from 40°C to 70°C. Previous studies along this thermal gradient revealed different patterns of hydrogen accumulation and methanogenesis (43, 51). It was, therefore, important to investigate fermentation in microbial populations found over this temperature range. Acetate and propionate were the major fermentation products to accumulate at all temperatures (Figure 3). Acetate accumulated to a higher concentration at the higher temperatures, whereas propionate accumulated to a higher level at the lower temperatures. Profound differences in the rates of accumulation of major fermentation products were not observed.

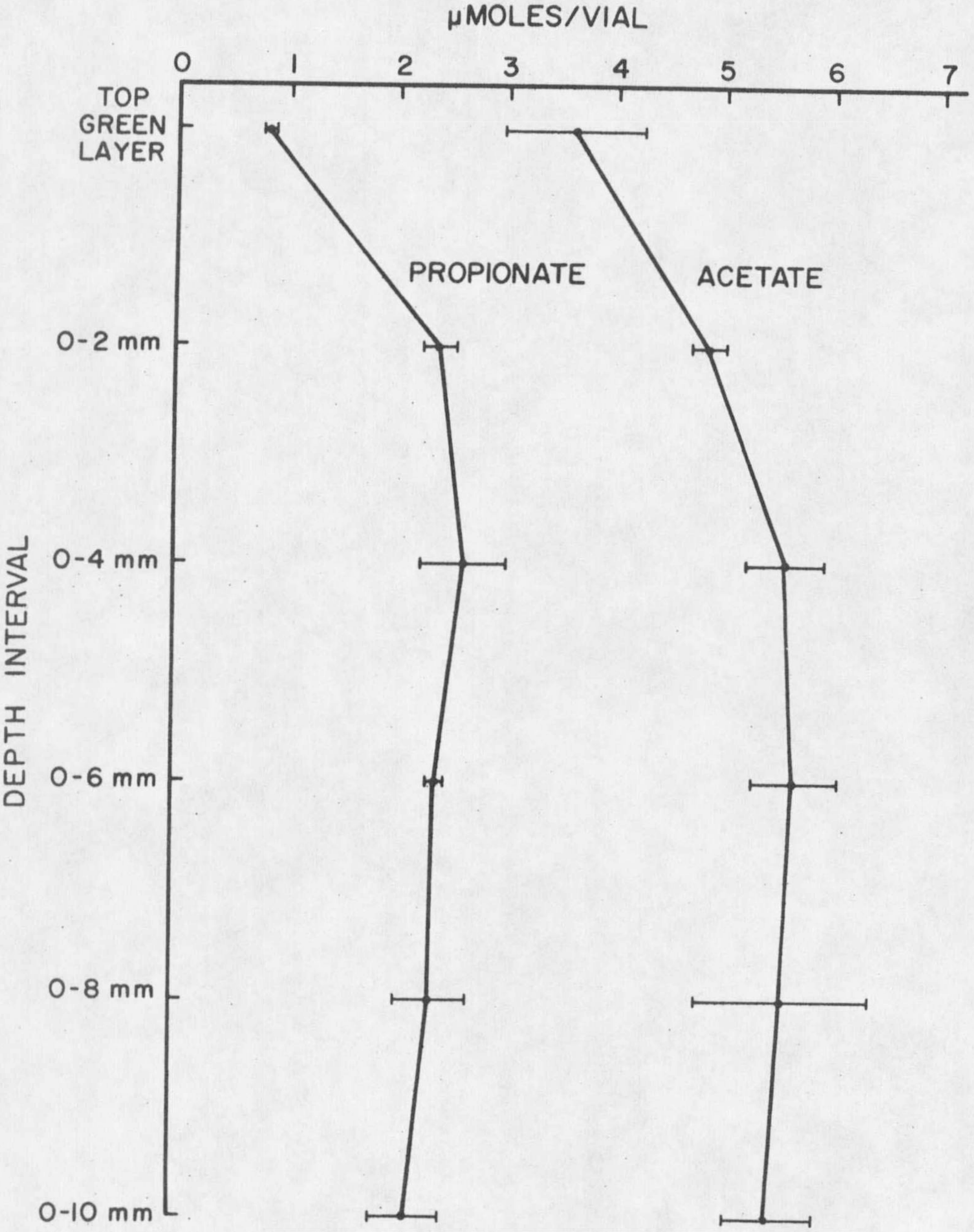

Figure 2. Depth profile of acetate and propionate accumulation after a 54 h dark anaerobic incubation of mat samples from Octopus Spring 55°C. Bars are standard error (n=3).

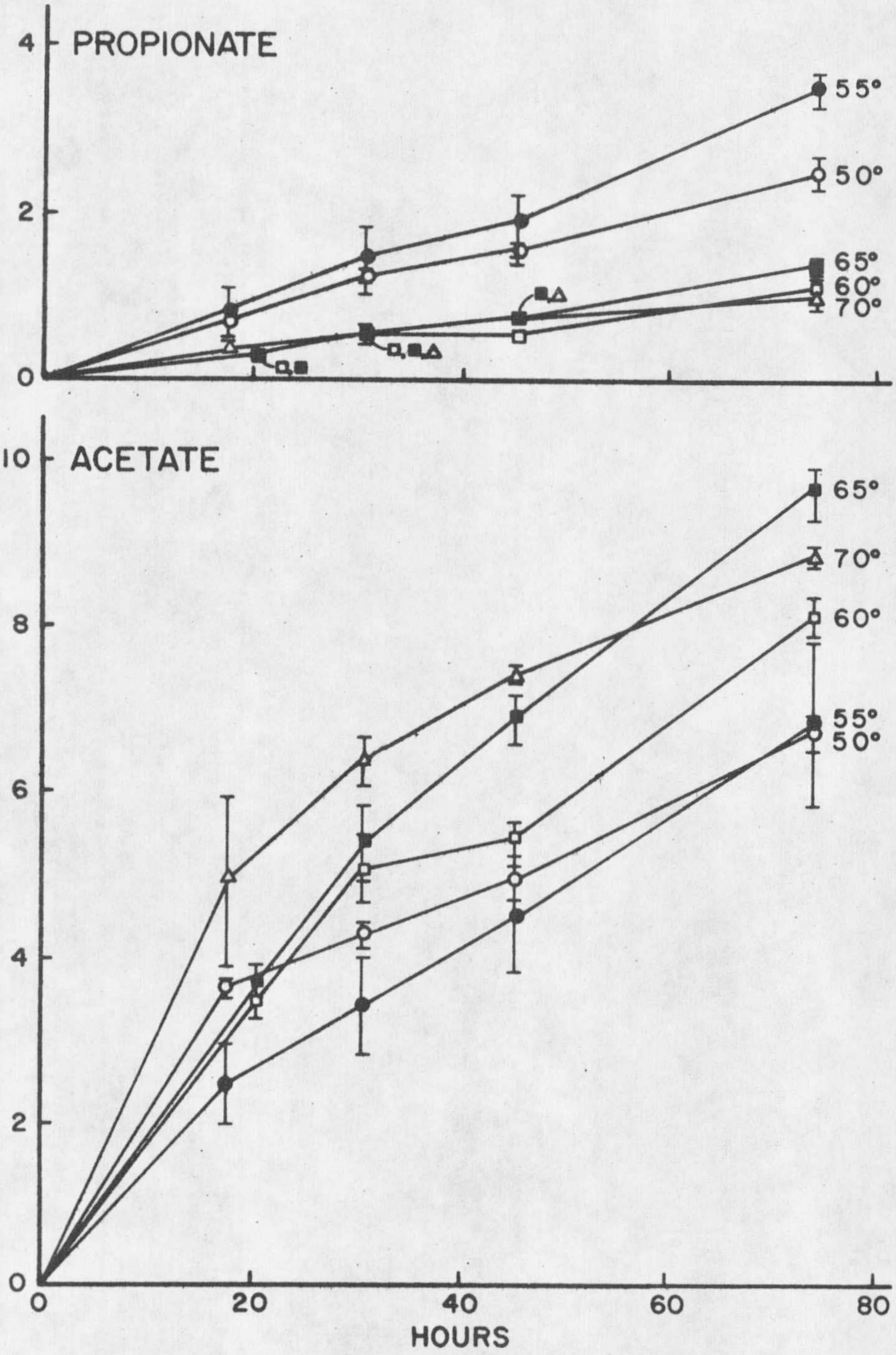

Figure 3. Acetate and propionate accumulation in mat samples collected at various temperatures in Octopus Spring. Bars are standard error (n=4).

Factors Affecting Product Accumulation

Comparisons of High and Low Hydrogen Sites

According to the interspecies hydrogen transfer model, the partial pressure of hydrogen in a system affects the types of fermentation products produced. Ward (51) found more hydrogen at a 65°C site in Octopus Spring than at a 55°C site. Core samples taken from both a 65°C and a 55°C site were incubated under dark anaerobic conditions. Hydrogen accumulated at 65°C whereas methane accumulated at 55°C (Figure 4). Acetate and propionate were the major fermentation products (Figure 5). Acetate accumulated to higher level at 65°C whereas propionate was higher at 55°C. A comparison of other fermentation products at the two temperatures showed that the level of other VFA's was higher at 65°C (Figure 6). Ethanol also accumulated. Lactate accumulation was observed at 65°C, although the rate of accumulation was less in comparison to acetate and propionate (data not shown).

Since the degree of fermentation may vary between these two sites, the ratio of each fermentation product to the major product, acetate, should more accurately reflect the importance of any product. Iso-butyrate,

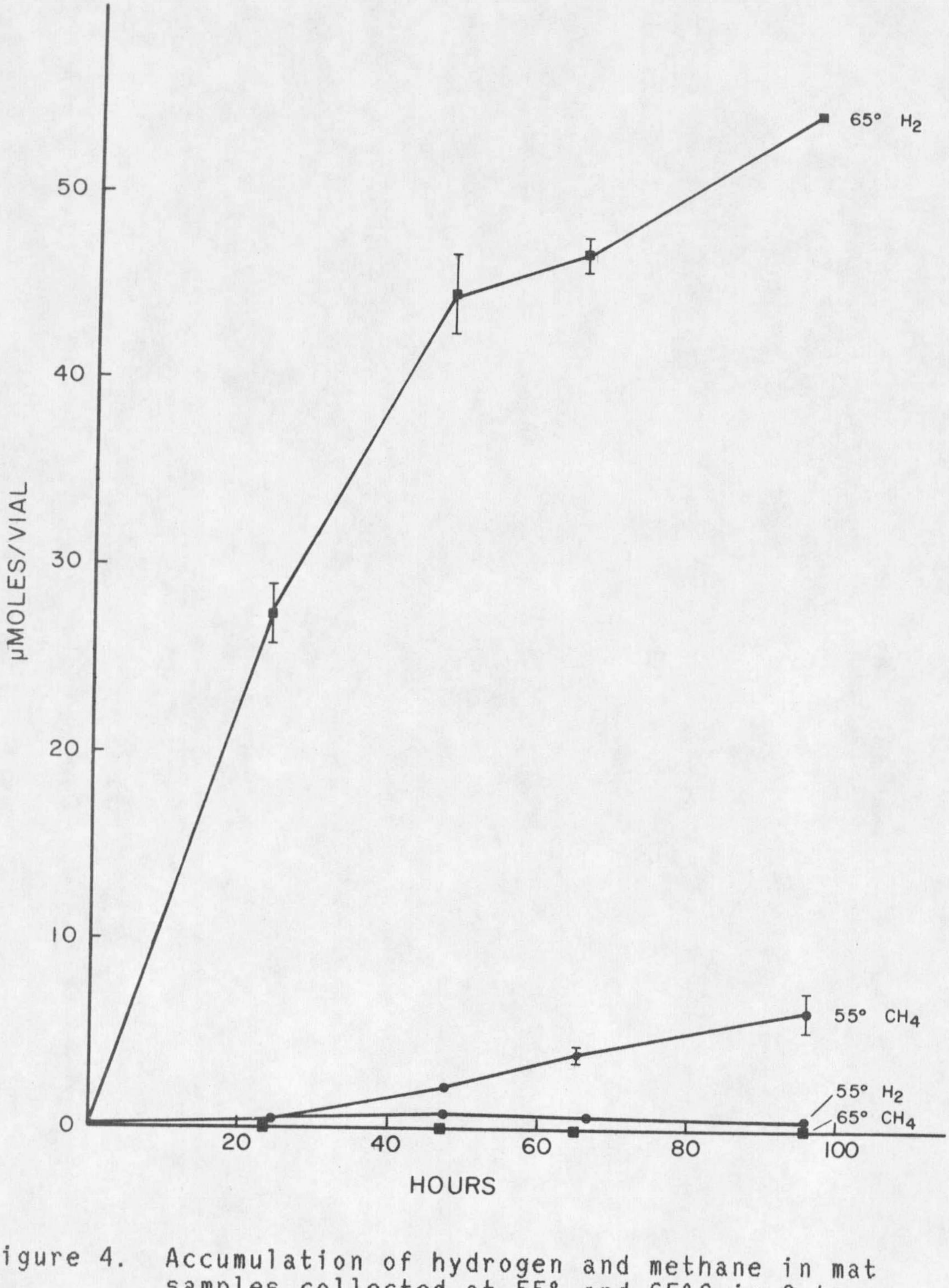

Figure 4. Accumulation of hydrogen and methane in mat samples collected at 55° and 65°C in Octopus Spring. Bars are standard error (55°, n=9, first two points, remainder n=4; 65°, n=2).

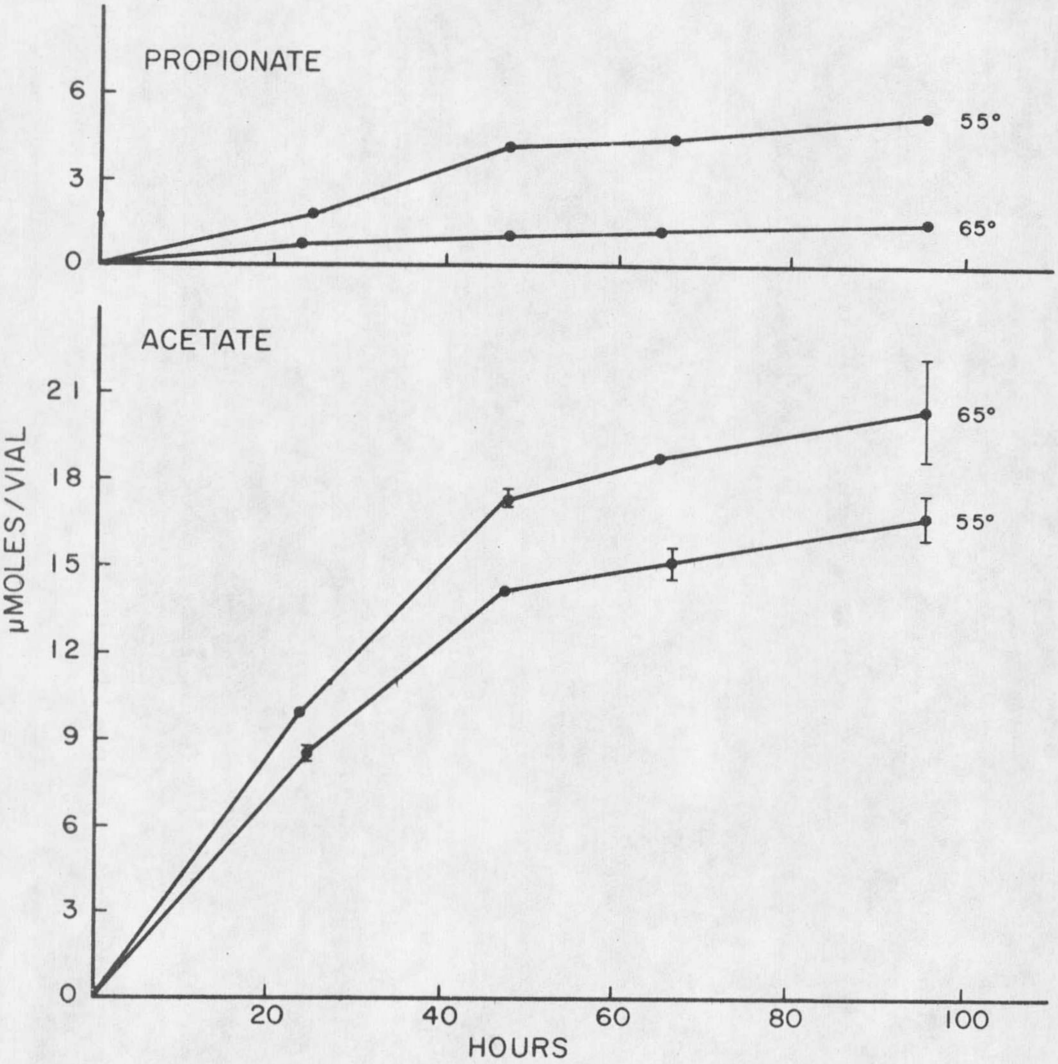

Figure 5. Accumulation of acetate and propionate in mat samples collected at 55° and 65°C in Octopus Spring. Bars are standard error; sample size noted in Figure 4.

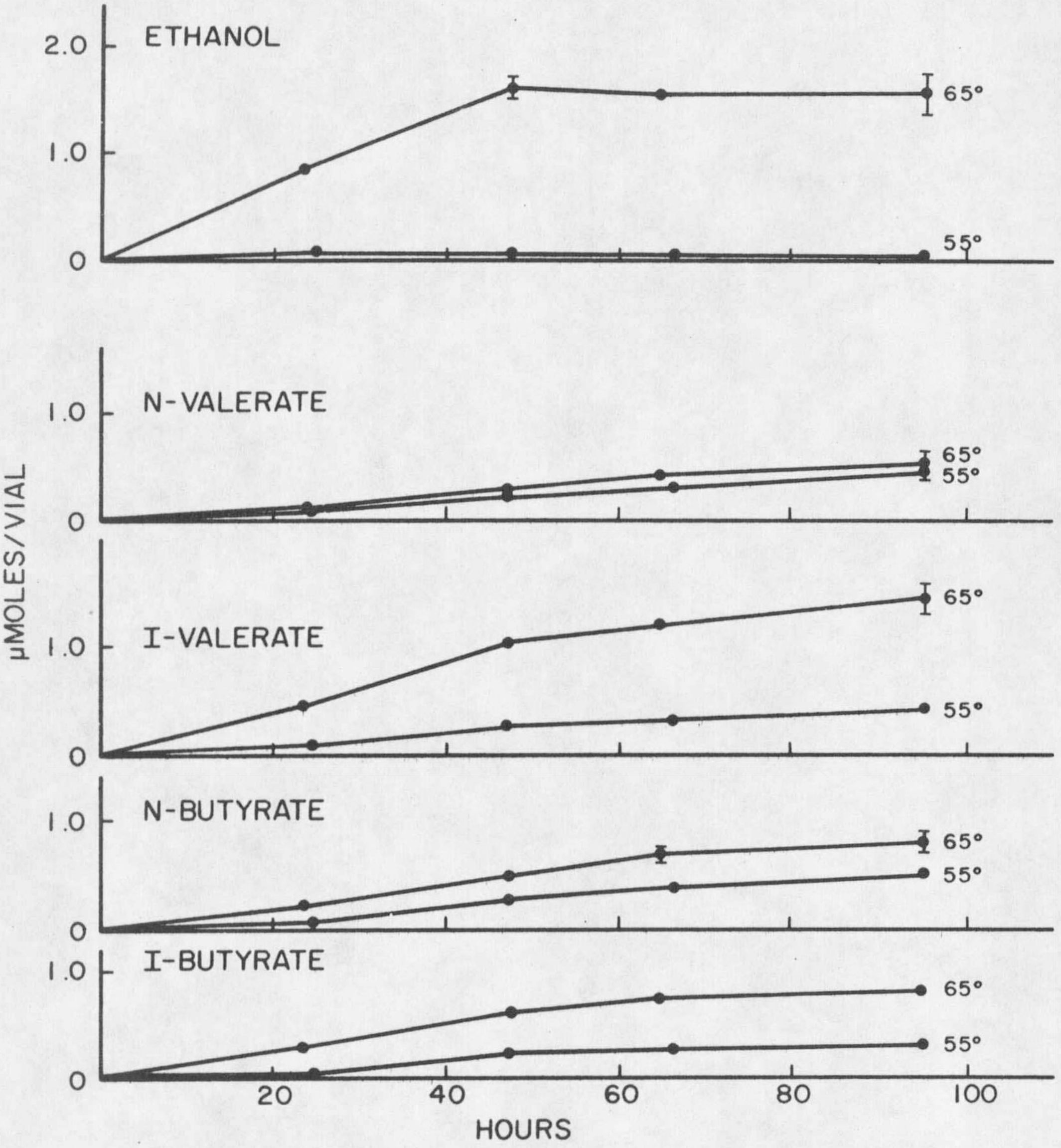

Figure 6. Accumulation of other fermentation products in mat samples collected at 55° and 65°C in Octopus Spring. Bars are standard error. Sample size noted in Figure 4.

iso-valerate, and ethanol accumulation relative to acetate was greater at 65°C than at 55°C (Table 2).

Table 2. Ratios of fermentation products to acetate produced after a 96 h dark anaerobic incubation of 55° and 65°C Octopus Spring mat.

CONDITION	ACETATE	PROPIONATE	I-BUTYRATE	N-BUTYRATE	I-VALERATE	N-VALERATE	ETHANOL
55°	---	0.310	0.017	0.029	0.025	0.028	0.004
65°	---	0.074	0.039	0.039	0.069	0.025	0.076
RATIO AT 65° / RATIO AT 55°	---	0.24	2.29	1.34	2.76	0.89	19.00

Artificial Increase in Hydrogen

To further test the effect of an elevated hydrogen level on fermentation product accumulation, 2-bromoethanesulfonic acid (BES) was added to artificially induce a high hydrogen environment. The data in Figure 7 showed that control cores exhibited methanogenesis with little hydrogen accumulation, whereas those incubated with BES showed an inhibition of methanogenesis and an increase in hydrogen. VFA analysis (Figure 8) revealed little difference in acetate accumulation patterns between samples containing BES and controls. Propionate (Figure 8) as well as iso-butyrate, n-butyrate, iso-valerate, n-valerate, and ethanol were higher in the samples containing BES (Figure 9). Table 3 shows that

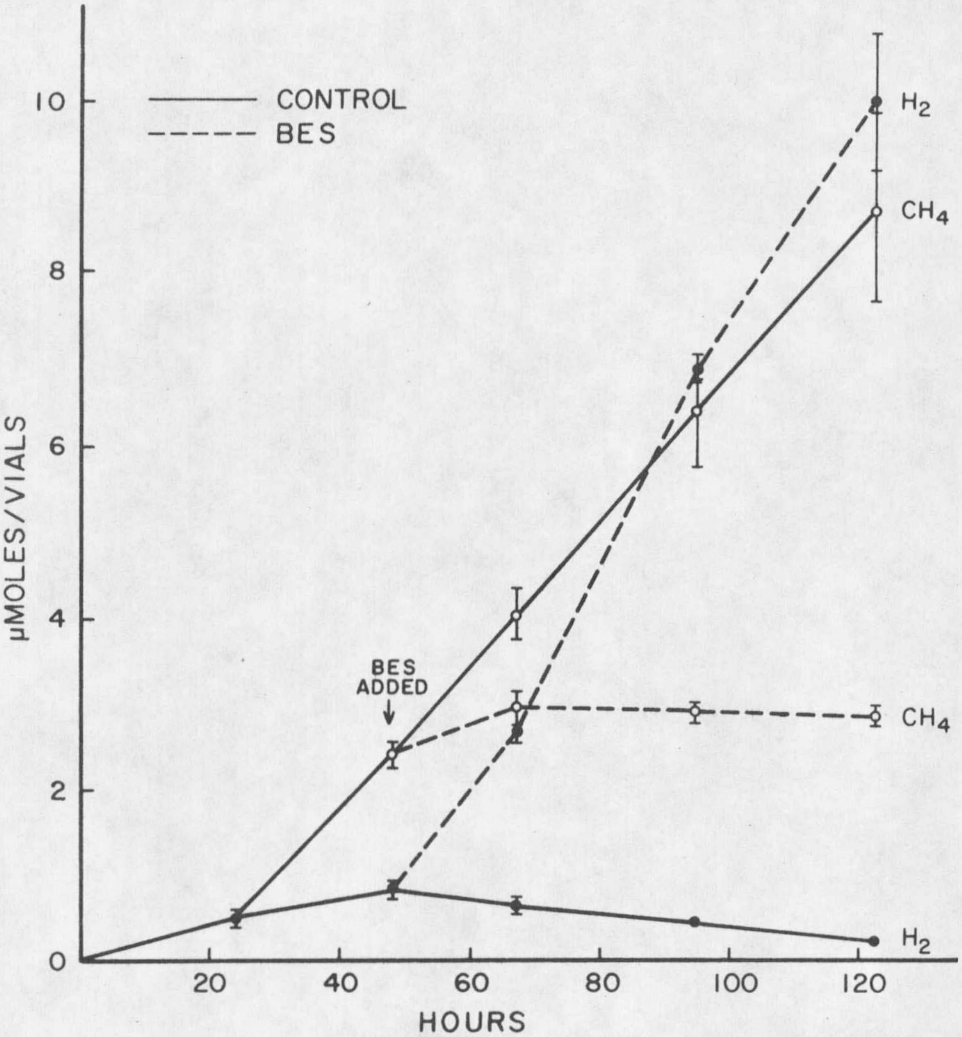

Figure 7. Accumulation of hydrogen and methane in the presence and absence of 2-bromoethanesulfonic acid (BES) in samples from a 55°C Octopus Spring site. Bars are standard error. Sample size for 55°C noted in Figure 4; n=5 for BES samples.

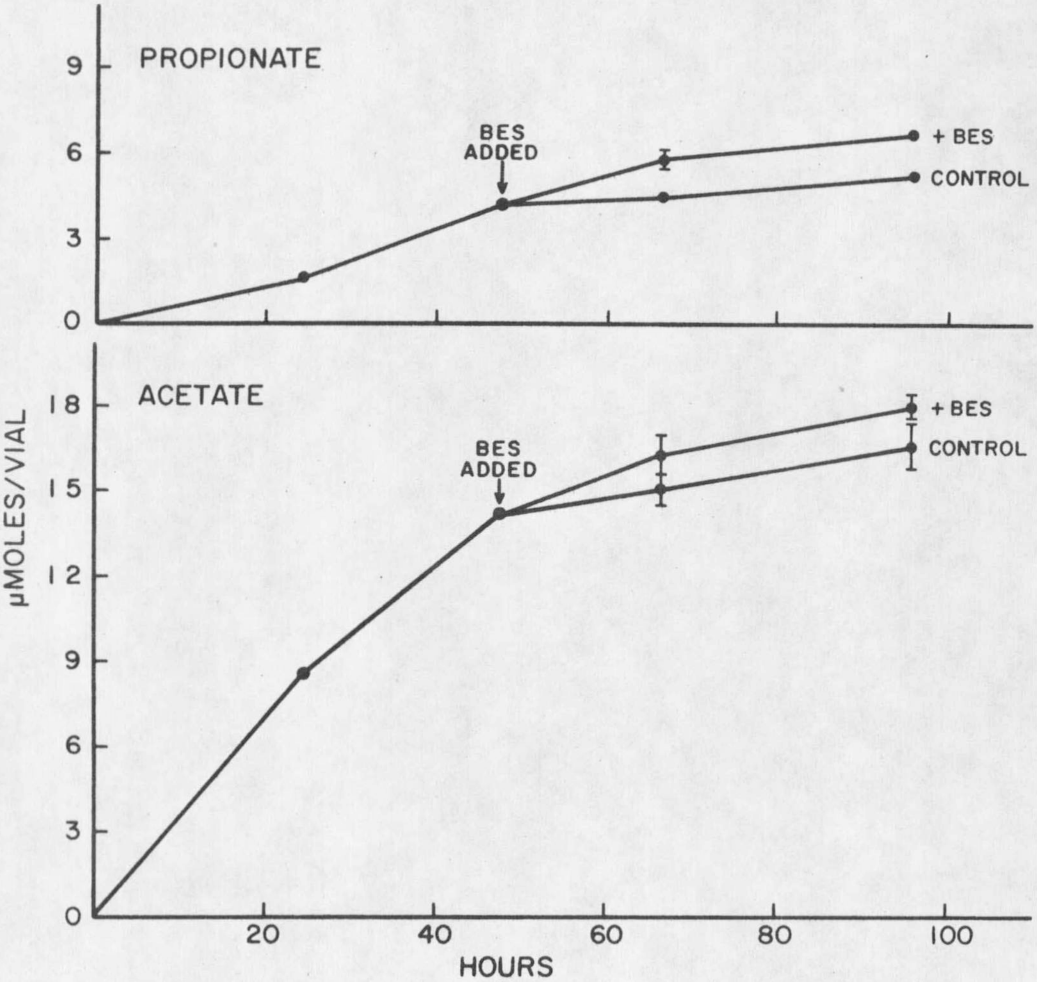

Figure 8. Accumulation of acetate and propionate in the presence and absence of 2-bromoethanesulfonic acid (BES) in samples from a 55°C Octopus Spring site. Bars are standard error. Sample size noted in Figure 7.

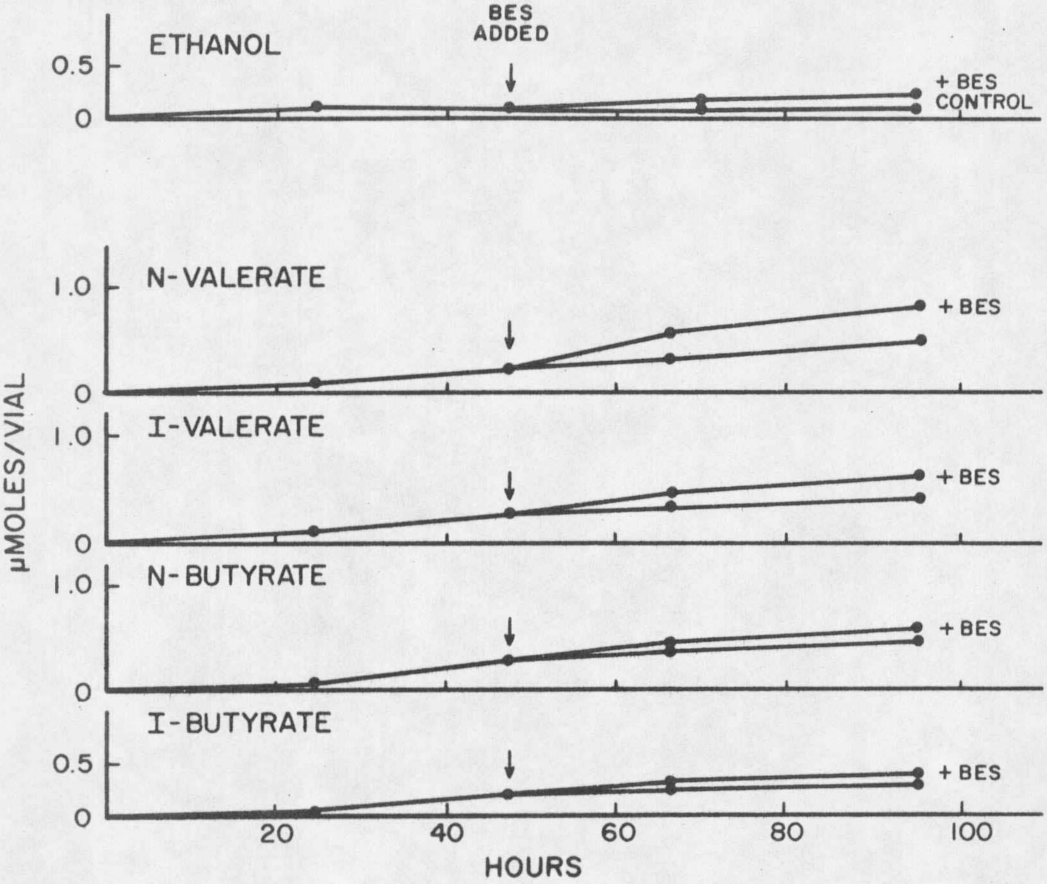

Figure 9. Accumulation of other fermentation products in the presence and absence of 2-bromoethanesulfonic acid (BES) in samples from a 55°C Octopus Spring site. Bars are standard error. Sample size noted in Figure 7.

n-valerate and ethanol accumulated to a greater level relative to acetate in the BES samples versus the controls. Lactate did not accumulate in BES samples (data not shown). In a subsequent BES experiment the incubation time was tripled and the hydrogen level had doubled by the end of the experiment. The only reproducible effect of hydrogen was an increase in ethanol accumulation in the BES samples relative to the controls (data not shown).

Table 3. Ratios of fermentation products to acetate produced after a 95 h dark anaerobic incubation of 55°C Octopus Spring mat in the presence and absence of 2-bromoethanesulfonic acid (BES).

CONDITION	ACETATE	PROPIONATE	I-BUTYRATE	N-BUTYRATE	I-VALERATE	N-VALERATE	ETHANOL
55°	---	0.310	0.017	0.029	0.025	0.028	0.004
+BES	---	0.377	0.020	0.031	0.033	0.046	0.013
$\frac{\text{RATIO AT +BES}}{\text{RATIO AT 55°}}$	---	1.22	1.18	1.07	1.32	1.64	3.25

Effects of Light

Cores from the 55°C Octopus Spring mat were incubated in situ under both dark and sunlight conditions for 10 hours and were subsampled for fermentation products at the end of the incubation period. Acetate accumulated to a higher level during dark incubation than in sunlight (Table 4). The level in the dark was higher than that in formalin controls suggesting that fermentation product accumulation is sensitive to light or other factors controlled by light. Formalin control data showed that at the time of sample collection, the level of acetate in the mat was higher than after a 10 hour incubation in the light; this indicated that acetate consumption occurred. Two other experiments performed on samples from a 55°C microbial mat at Mushroom Spring showed a similar trend of greater acetate accumulation in the dark versus the light (Table 4).

Table 4. Effect of light on acetate accumulation in Octopus Spring and Mushroom Spring 55°C mat samples. * indicates significant differences ($p \leq 0.05$) of dark samples compared to light samples.

SAMPLING SITE	CONDITION	COLLECTION TIME	ACETATE (μMOLES/VIAL ± SD)
Octopus Spring	Formalin Control	0835	0.95 ± 0.57
	Light (10 Hr. incubation)	0835	0.04 ± 0.02
	Dark (10 Hr. incubation)	0835	1.74 ± 0.05*
Mushroom Spring experiment # 1	Light (6 Hr. incubation)	1200	0.17 ± 0.07
	Dark (6 Hr. incubation)	1200	0.45 ± 0.18*
Mushroom Spring experiment # 2	Light (6 Hr. incubation)	1200	0.17 ± 0.14
	Dark (6 Hr. incubation)	1200	0.78 ± 0.09*

Population Potential for Uptake of Fermentation Products

Since the accumulation of ethanol and other products was higher at 65°C than at 55°C, activity measurements of the higher temperature population were made to determine if the mat microorganisms were adapted to take up more reduced fermentation products. ^{14}-C labeled acetate, propionate, butyrate, lactate, and ethanol were tested in the 1-3 mm interval of the mat. This segment was demonstrated by Tayne (48) to have a maximum for uptake and metabolism for several radiolabeled compounds tested at a 55°C site. Data for the 65°C heterotrophic potential experiment are reported on a modified Lineweaver-Burk plot in Figure 10. (Note that all data are not reported on this plot. See Table 6 in the Appendix for a listing of all the data.) The inverse slope (V_{max}) of each compound was determined from linear regression analysis (Table 5). The microbial community at 65°C had a greater potential for acetate and lactate metabolism than for the other compounds tested. The potential for ethanol metabolism was greater than for fatty acids other than acetate and lactate.

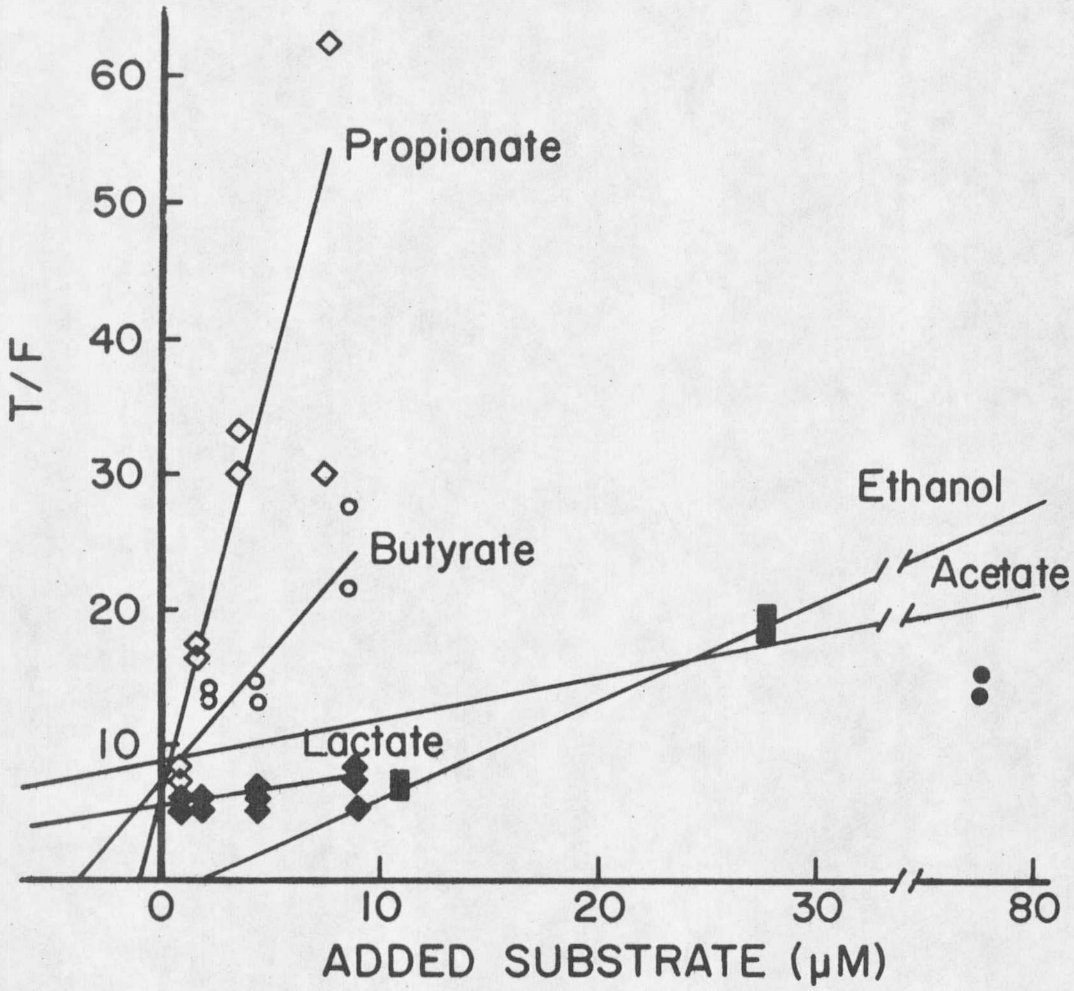

Figure 10. Modified Lineweaver-Burk plot of the uptake and metabolism of ^{14}C-fermentation products in the 1-3 mm interval of the 65°C Octopus Spring mat. T/F is the incubation time divided by the fraction of label metabolized (μCi metabolized/μCi added). Note data all points are not shown. See Table 6 for a complete listing of data.

Table 5. V_{max} for uptake and oxidation to $^{14}CO_2$ of ^{14}C-fermentation products in the 1-3 mm interval of Octopus Spring 65°C mat. Units of V_{max} are µmoles of substrate incorporated/l/h. r is the correlation coefficient for a straight line derived from a linear regression of all data points for each compound.

COMPOUND	V_{MAX}	r
ACETATE	6.97	0.99
PROPIONATE	0.17	0.90
BUTYRATE	0.52	0.93
ETHANOL	1.37	0.99
LACTATE	4.55	0.69

DISCUSSION

Studies on anaerobic processes in the Octopus Spring microbial mat showed active methanogenesis and little acetogenesis. As little was known about fermentation in this system, the goal of this research was to further study decomposition of the Octopus Spring microbial mat by investigating what fermentation products accumulated in the mat, where they accumulated within the mat, and what factors affected their accumulation.

Acetate and propionate were the predominant fermentation products to accumulate at a 55°C site. They accumulated in a ratio of about 3:1. This agrees well with the importance of acetate as a decomposition product in many other anaerobic environments (54). Acetate was reported the major fermentation product in lake sediments (29). In Lake Wintergreen sediments, the relationship of the two major fermentation products was similar with a ratio of 4.7:1 for acetate to propionate (25). Both acetate and propionate were the dominant fermentation products found in 40°C and 60°C cattle waste digesters (32) as well as in a high sulfate salt marsh (3) and marine sediments (47). The low level of importance of other volatile fatty acids in Octopus Spring and sediments was also comparable (3, 29, 32).

It is important to keep in mind that the methods incorporated in my studies involved measuring the accumulation of fermentation products and not their turnover by the natural populations. A number of workers, (3, 29, 64) investigated the kinetics of fermentation product degradation and their subsequent contribution to methanogenesis or sulfate reduction in natural systems. The importance of a product in a system may be underestimated by simply viewing accumulation data alone. For example, lactate does not accumulate in 55°C mat samples, and yet Tayne (48) showed that this compound is metabolized by the microbial populations present under dark and light, aerobic and anaerobic incubation conditions. Lactate, then is probably an important fermentation substrate to the microbial mat community.

To further understand anaerobic decomposition in the Octopus Spring mat, the location of fermentation was investigated. Doemel and Brock (17) speculated that decomposition processes occurred in the lower portion of the mat, below the photic zone. Revsbech and Ward (40) demonstrated that the mat was superoxic in the upper 3 mm during the daylight hours with anoxic conditions existing during darkness. Since fermentation is an anaerobic process, one would expect fermentation to occur either in the layers which are always anoxic (below 7 mm) or only at night closer to the mat's surface.

The location of anaerobic decomposition in the Octopus Spring mat was studied by incubating subsections of 1 cm cores under dark anaerobic conditions. That the level of fermentation products did not increase when core sections greater than 4 mm were incubated indicates that the majority of fermentation occurs within the upper 2-4 mm of the mat. This finding relates well to the vertical distribution of other anaerobic processes in the hot springs. Ward (51) showed that rate of methane production peaked 1 mm below the surface and decreased with depth in a 45° and a 55°C Octopus Spring. At a 65°C site, hydrogen production, and not methanogenesis, was higher in the upper mat. In the high sulfate hot spring, Bath Lake, sulfate reduction was higher in the top 5 mm interval than in the deeper layers of the mat (53).

In studies on decomposition in anaerobic sediments, it has been repeatedly reported that highest rates of anaerobic decomposition are near the sediment surface (54). Depending on the particular system of study, either methanogenesis (29, 64) or sulfate-reduction (3, 30) dominates in the upper sediment interval, and decreases with depth. Turnover studies of acetate in low and high sulfate systems also reflect greatest rates of turnover at the sediment surface (3, 29).

That fermentation occurred in the upper 4 mm of the mat has interesting implications with respect to daily fluctuations in oxygen concentration. The mechanism of survival for decomposition organisms, which in the top 4 mm of the mats are alternately exposed to superoxic and anoxic conditions, is unknown. Some may be facultative anaerobes able to survive the diurnal changes in oxygen levels. All of the fermentative isolates from Octopus Spring are anaerobes (52); at least four are considered obligate anaerobes. However, one isolate Thermoanaerobacter ethanolicus, was not killed during a one hour incubation in aerobic media, but grew only under anaerobic conditions (59). Another isolate, Clostridium thermohydrosulfuricum, was also shown to survive for several days in aerated medium at 60°C without growth (57).

It is possible that in the natural mat environment some anaerobic organisms may be more tolerant to oxygen than microorganisms exposed to constant anoxic conditions. Revsbech and Ward (40) isolated a methanogen from the upper layers of the mat which was rather insensitive to oxygen. This is unusual since methanogens, as a group, are considered strict anaerobes (33). Oxygen concentrations, however, are not the only important parameter when considering anaerobic environments. The Eh, the oxidation-reduction potential, is also a major factor which determines whether or not certain organisms will grow. The cynanobacterium, Oscillatoria terebriformis, grows as a photoheterotroph and a dark heterotroph only when the redox potential is less than -100 mV. An oxygen-free environment is not sufficient for growth (42). Methanogens require a low potential of less than -300 mV (33). It would be useful to determine Eh profiles in the Octopus Spring mats, and compare these with diurnal fluctuations of oxygen.

Microniches in the top of the mat could allow these organisms to survive the extreme diurnal oxic fluctuations. The gelatinous nature of the mat may hinder oxygen diffusion just as extracellular polysaccharides appear to limit diffusion of nutrients and other molecules to bacteria encased in biofilms. Current techniques, however, are not sensitive enough for measuring oxygen gradients surrounding a single bacterial cell.

As an alternative to oxygen tolerance, organisms may adjust their placement in the mat with changes in oxygen concentration. There are several examples of how motile organisms position themselves in mats in relation to chemical gradients. _Oscillatoria terebriformis_ remains on the surface of the microbial mat during most of the day and at night migrates 1-2 mm down into the mat where conditions are anaerobic (41). Jorgensen and Revsbech (24) showed that _Beggiatoa_ spp. formed layers of cells at the interface of oxygen and hydrogen sulfide in a mat from a marine sediment. Nelson and Jannasch (38) demonstrated in laboratory agar cultures that the growth of a marine Beggiatoa isolate depended on its preference for reduced oxygen concentrations and a limited sulfide concentration in combination with gliding motility. In the Octopus Spring ecosystem, Doemel and Brock (17) observed Chloroflexus to glide to the mat's surface during darkness. Chloroflexus can only grow as an aerobe in the darkness.

If the mat organisms do adjust their placement within the mat due to changes in oxygen gradients, it should be noted that four of the five fermentative isolates have flagella (58, 59, 60, 78) that could support such motility. Some methanogens, such as a strain of Methanosarcina barkeri isolated from an enrichment of sewage sludge and a Methanothrix species isolated from a 58°C digester, have gas vacuoles (1, 79). Although Methanobacterium thermoautotrophicum, isolated from Octopus, was not observed to have gas vacuoles, it is possible that other methanogens not isolated could adapt to diurnal fluctuations by using such structures.

Light influenced the accumulation of fermentation products. Samples incubated in the light had less acetate than corresponding samples incubated in the dark. Formalin controls taken at zero time (early morning) and at 5 hours into the experiment revealed higher concentrations of acetate than in samples incubated in the light for

10 hours. (Formalin controls for in situ acetate measurements at 10 hours were not taken.) This suggested that light-dependent metabolism of acetate occurred. Tayne (48) showed that fermentation products were taken up under light aerobic conditions by a filamentous bacterium, shown to be Chloroflexus aurantiacus by a combined immunofluorescence-autoradiographic procedure. As few of these fermentation products were metabolized in the dark, it is likely that these compounds are taken up by this photoheterotroph during the day.

Light-driven photosynthesis not only affects oxygen concentrations but pH conditions as well. Using microelectrodes in a 55°C mat, Revsbech and Ward (40) found the lowest pH value to be present before sunrise in the 1 to 6 mm depth interval. The photosynthetic uptake of bicarbonate during the day as well as fatty acid production at night are thought to influence the pH change from basic during the day towards more acidic at night (40). The observations of: a higher acetate level in the morning mat, the anaerobic and acidic conditions of the night mat, and my continual observations of fermentation product accumulation in core samples incubated under dark anaerobic condition support active fermentation in the Octopus 55°C mat at night.

Fermentation occurred in samples collected along the thermal gradient from 70° to 50°C. The dominant fermentation products were always acetate and propionate. Ward (51) showed that methane production in situ occurred from 68° to 30°C with very little methanogenesis observed in the 68° to 63°C range. Hydrogen was noted to accumulate in the 65°C mats. Carbon dioxide and hydrogen, and not acetate, were found to be the important methane precursors over a 60° to 45°C range in the mats (43). These observations support the model proposed for anaerobic decomposition in which both fermentative and methane producing bacteria play important roles. In Octopus Spring, hydrogen and carbon dioxide produced during fermentation are used by the methanogens and Chloroflexus is implicated in the photoincorporation of acetate.

Zeikus, et al. (76) speculated that fermentative anaerobic bacteria that produce hydrogen may function in nature at temperatures greater than 80°C but that methanogens may not. Experiments revealed that hydrogen formation but not methanogenesis was detected at 80°C during anaerobic decomposition of the Octopus Spring mat. The 65°C mats provided a natural system in which to investigate the effects of hydrogen on fermentation production as related to the interspecies hydrogen transfer theory. Samples from a 65°C community showed different patterns of fermentation product accumulation than did those from a 55°C environment. Analysis of fermentation products in this higher hydrogen environment (65°C) showed a greater ratio of reduced products to acetate, as specified by the the interspecies hydrogen transfer model. The finding of a high ratio for ethanol accumulation correlates well with four of the fermentative isolates which produce ethanol. Thermophilic isolates from hot springs may prove useful for industrial production of chemicals, such as ethanol (57).

The lower propionate accumulation at 65°C, however, was not expected. In many other systems studied, such as the rumen or anaerobic digester, when methanogenesis ceased, a higher hydrogen level caused propionate to accumulate (34). The increase in hydrogen concentration resulting from the inhibition of methanogenesis either inhibits propionate metabolism by acetogenic bacteria or forces fermentors to produce more reduced fermentation products due to the breakdown in interspecies hydrogen transfer. It was not probable that acetogens broke down the propionate at 65°C, due to the high inhibitory concentration of hydrogen at this temperature. Tayne (48) showed little evidence for propionate acetogenesis even at lower hydrogen concentrations.

Another approach in studying the effect of hydrogen was to inhibit methane production in the 55°C mat. Measurements of samples incubated in the presence and absence of an inhibitor of methanogenesis, 2-bromoethanesulfonic acid (BES), showed substantial differences in the ratio of fermentation products to acetate only in ethanol accumulation. It should be noted that the hydrogen level during this experiment did not reach that observed after incubating 65°C samples and possibly was not high enough to induce production of reduced products. Tayne (48) found that butyrate acetogenesis was sensitive in a BES experiment. Acetogenesis, however is much more sensitive to hydrogen concentration than is the production of reduced fermentation products.

There are limitations in only viewing fermentation product accumulation and assuming that the resulting products are important to a microbial community. As noted before, some compounds produced in the system may be metabolized, and therefore, will not accumulate. To further understand the importance of compounds in the hot springs, photoheterotrophy was used as a bioassay of what fermentation products the microbial population were adapted to use. These heterotrophic potential results represent a potential of the microbial population to take up and metabolize quantities of substrate thought to saturate the organisms' permease systems.

Acetate, lactate, and ethanol had a greater potential for uptake by the community at 65°C while propionate and acetate had the greatest potential for incorporation at a 55°C site. Lactate accumulated in the 65°C system, if only slightly, but did not accumulate in the 55°C site.

Tayne (48) showed that lactate was metabolized at 55°C under dark, anaerobic conditions as well as under light aerobic and anaerobic and dark aerobic conditions. It was not determined if lactate was metabolized at 65°C.

Propionate accumulation at 55°C was higher than at 65°C. Again, experiments were not performed to determine whether propionate was metabolized at 65°C, but Tayne (48) found that it was only partially metabolized under dark anaerobic conditions at 55°C. The V_{max} value for propionate uptake at 55°C was the highest for all compounds tested; at 65°C the V_{max} for propionate was the lowest. The relative decrease in propionate accumulation at 65°C may reflect the population's inability to produce as much propionate as was seen at 55°C. As mentioned above, acetogenesis of this compound was highly unlikely, as a high hydrogen level inhibits the activity of these organisms. Whatever the reason, the decrease in propionate levels at the higher temperature coincided with a population which is not adapted to taking it up.

The Octopus Spring microbial mat is an interesting ecosystem for study of natural microbial interactions. We are limited, at this point, in understanding community relationships by not knowing the identities and pure culture activities of other bacteria in this system. By studying gross processes, however, we have learned much about important factors which influence the microbial population.

Fermentation is an active process in Octopus Spring. Acetate and propionate are the major fermentation products that accumulate under dark anaerobic conditions. Fermentation products accumulate predominately in the upper 4 mm of the mat. This location of this process has raised some interesting questions concerning interactions of the community's population. For example, how do fermentative organisms adapt to the diurnal changes in oxygen concentration? The observation that mat samples incubated in the light had less accumulation of fermentation products than corresponding dark samples integrates well with Tayne's finding that Chloroflexus photoincorporated fermentation products (48). As Tayne pointed out, this system resembles the rumen in that the further breakdown of fermentation products is not an important process. Instead, these products are cycled for use by Chloroflexus; in the rumen, they are cycled for direct use by the animal. Also in both the rumen and in the Octopus Spring mats, acetate is not an important methane precursor.

That interspecies hydrogen transfer is active in the 65°C environment is supported by the shift in the accumulation of more reduced fermentation products, with the exception of propionate, at this higher temperature compared to 55°C. The importance of these reduced products may be reflected in the heterotrophic potential results. The population at 65°C was more adapted to taking up lactate and ethanol than was the community at 55°C. Thus, at a 65°C mat with less methanogenesis and more production of hydrogen than the 55°C system, the importance of reduced fermentation products agrees with the theory of interspecies hydrogen transfer and adaptiveness of the bacterial community for these compounds.

Fermentation in Octopus Spring thus not only appears to serve as a means of anaerobic decomposition in a low sulfate environment in which methanogenesis and little acetogenesis occur, but it also appears to be important in supplying the photoheterotroph, Chloroflexus, with nutrients.

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APPENDIX

Table 6. Results of heterotrophic potential experiment to determine V_{max} for uptake and metabolism of fermentation products in the 1-3 mm interval of Octopus Spring 65°C mat.

^{14}C-fermentation product	A (µmoles)	T/F (hours)
acetate	77.78	14.28
		15.15
		15.62
	171.21	33.33
		35.71
		35.71
	354.55	62.50
		71.43
		71.43
	825.76	125.00
		125.00
		125.00
propionate	0.87	7.14
		8.20
		8.33
	1.80	16.67
		17.24
		17.24
	3.56	31.25
		33.33
		33.33
	8.13	31.25
		62.50
		62.50

Table 6. Continued

^{14}C-fermentation product	A (µmoles)	T/F (hours)
butyrate	1.07	7.14
		7.81
		8.09
	2.17	13.51
		13.98
		14.29
	4.20	13.51
		14.71
		14.71
	8.64	21.74
		21.74
		27.78
lactate	1.06	5.26
		5.38
		6.17
	1.94	5.75
		5.95
		6.02
	4.34	5.62
		5.81
		6.85
	8.90	5.74
		7.94
		8.47
ethanol	10.94	6.85
		7.69
		7.69
	27.75	18.52
		18.52
		20.00

Table 6. Continued.

^{14}C-fermentation product	A (µmoles)	T/F (hours)
ethanol	56.70	38.46 38.46 41.67
	122.49	83.33 83.33 100.00

X