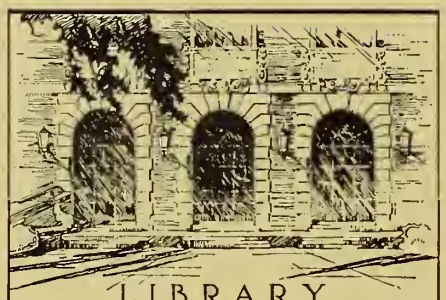

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BENTHIC SAMPLING, ANALYSIS, AND ECOLOGICAL STUDIES OF NEMATODES

By
K. Y. BALIGA

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ABSTRACT

An ecological study of benthic nematodes is important from the standpoint of having an understanding of the distribution of these organisms between the benthos and the overlying water. Under favorable conditions, the benthos may become a breeding site and contribute nematodes to the water; or nematodes may settle into the benthos from waste treatment plant effluents. The study reported herein deals with some of the ecological factors involved in the occurrence and abundance of nematodes in the stream benthos.

It is imperative that a satisfactory method of isolating nematodes from benthic samples be available for quantitative results to be obtained. Therefore, considerable time and effort were spent in developing a technique utilizing the centrifugal flotation principle.

Nematodes were found to be present in all sections of the Boneyard-Saline Branch stream system investigated. The concentration of nematodes in water and benthic samples were significantly higher below the outfall of the Urbana-Champaign waste treatment plant than above the outfall. The concentrations of nematodes in the benthos during October through November, 1963, were found to be about twice that observed during June through August, 1963. This was attributed to the more favorable temperatures during October-November, 1963. During the same period, the nematode concentration in the water of the stream increased 17 times, showing that the concentration in the stream benthos did not bear any constant ratio to that in the water. The nematodes in the benthos were severely affected by high flows in the stream because of the

erosive action of such high discharges. The concentration of nematodes in the benthos was observed to increase during low flows. Dissolved oxygen in the stream water was found to be conducive to the growth of benthic nematodes as was the absence of currents. The variation found in physical properties such as effective size and uniformity coefficient and chemical properties such as chemical oxygen demand, total nitrogen, and pH of the bottom material were found to have no direct relationship to the nematode concentration. The nematode concentration in the benthos was observed to be higher in the littoral zone than in the channel zone, the reason being the absence of currents. About 70 percent of the nematodes were found to be in the top two centimeters of the benthos, where oxidation reduction potential measurements showed the absence of anaerobic conditions. The number of nematodes in the benthic samples obtained from the stream were found to decrease when exposed to temperatures substantially higher or lower than their natural environment, over a period of 10 to 14 days. However, another laboratory study showed that under a favorable temperature of 20°C, nematodes were found to increase in number in a bed of bottom material obtained from the stream. The presence of antibiotics in such a bed of bottom material, however, prevented any growth, probably due to the absence of bacterial food on which the nematodes feed in the natural environment.

The numbers within the parentheses in the text refer to the numbers of references listed in Bibliography.

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I. INTRODUCTION

GENERAL ECOLOGICAL RELATIONSHIPS

The simplest definition of ecology is that it is the study of the relationship between a living organism and its environment (1, 2, 3). The living (biotic) and the non-living (abiotic) environment has a definite effect on the abundance and behavior of an organism. The organism in turn adapts itself to the environment by a gradual process of evolution. This cause and effect phenomenon of the environment on the organism is experienced by every generation of organism but the process of evolution takes place after thousands of generations. A particular set of environmental conditions -- physical, chemical and biological -- constitute what is called an ecosystem. An ecological study is generally restricted to one ecosystem; the aim of such a study being the evaluation of the interrelationships existing within the ecosystem.

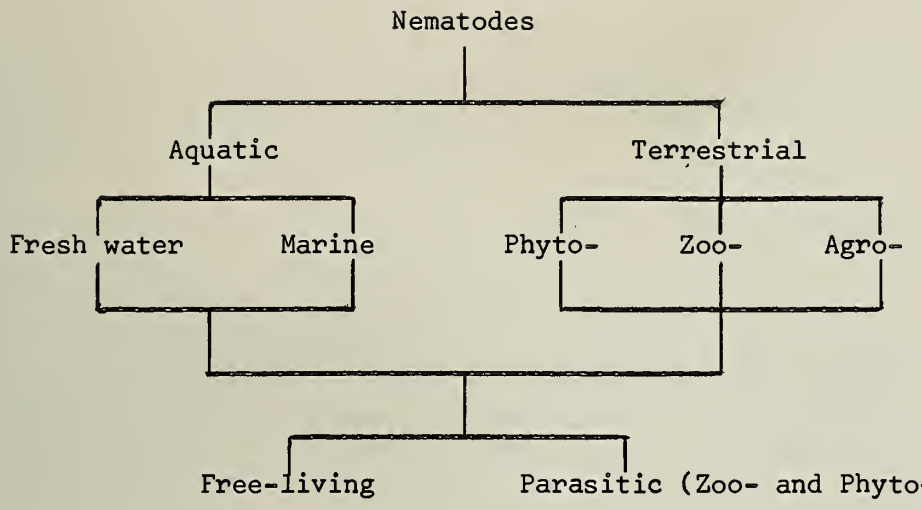
Every living organism in this world must perform the necessary functions of life in order to survive. A suitable environment for an organism must provide an adequate food supply for the organism to carry on its life processes of metabolism and reproduction. A change in the environment manifests itself with respect to the organism through changes in the population of the organism, feeding habits of the organisms, or in alterations of metabolic pathways. If the environmental changes are severe, gradual changes in morphological features of the organism may take place or the organism may even become extinct. Therefore, both the availability of essential materials needed for metabolism and growth and the adaptability of the organism to the new environmental conditions determine the occurrence, abundance and behavior of any organism in a given environment. Any factor which upsets this delicate balance between the organism and its environment, either through scarcity or overabundance, may seriously affect the occurrence

and abundance of that organism in a particular environment. Such a factor is called a "limiting factor" (3). The limiting factor can be physical, chemical or biological. The physical factors may be moisture, temperature, light, currents, or pressure. The chemical factors include the biogenic salts and gases available in the ecosystem. The biological factors are, tolerance, symbiosis, antibiosis, predation, parasitism and competition. It is obvious from the above broad spectrum of factors that the interrelationships and interdependencies can be very complex within an ecosystem.

An ecological study of an economically important organism can furnish valuable information by which control or exploitation of that organism can be achieved. The organisms under study in this investigation are called nematodes.

NEMATODES

Taxonomically, nematodes form a phylum, although earlier taxonomists placed them in a class of the phylum Aschelmenthes. Morphologically, these organisms are bilaterally symmetrical, cylindroid, unsegmented, with the anterior part tapering more than the posterior part (4, 5). Anatomically, they have a cuticle or a body-wall within which are the digestive system, the nervous system, the excretory system and the reproductory system. These organisms have a very characteristic locomotion in that they have an undulating swinging movement. These features generally suffice to identify the worms as nematodes. Reproduction in these organisms is generally bisexual. In addition to the most predominant dioecious species, hermaphroditic and parthenogenetic species can also be found. The length of these worms may be from a few microns to 3 to 4 mm, with the width varying from a few microns to about 250 microns. The larger sizes are less common, specially among the free-living aquatic species. Ecologically, the nematodes can be classified as follows:



As can be seen from the above classification, both free-living and parasitic forms of nematodes have been found on land and in water. As a matter of fact, they are such a variable group, that they have adapted themselves to most environments where life exists. The fact that they are ubiquitous points significantly to their tremendous ability to survive long periods of dormancy as well as to their ability to withstand adverse physical and chemical conditions. The prevalence, distribution, and importance of nematodes can best be expressed by quoting from two of the early workers (6). Bastian (7) reported in 1865 (as per reference 6),

> "As a result of my investigations, I am inclined to believe that these free nematodes will be found to constitute one of the most widely diffused and abundant groups in the whole animal kingdom, rivalling, in the first respect at least, the almost ubiquitous Diatomaceae. --- I have found (nematodes) in all specimens of soil examined, in moss, various species of lichen, about the roots of fungi, also the roots of grasses, and between the sheaths of their leaves, among the mud of ponds and rivers, on the fresh water algae, amidst decaying liverworts and mosses, and on submerged aquatic plants. The marine species exist in great abundance in the surface mud of rivers and estuaries, in the sand, and among the small stony debris under the shelter of rocks, as well as in the tide pools, where they swarm about the roots of the corallines and on some of the smaller and finer seaweeds, especially those having a dingy appearance from the

> presence of Diatomaceae. And lastly, two or three species I have found in the greatest abundance, as pseudo-parasites, within the substance of some of the softer sponges. So numerous are they in these latter situations, that it is rather surprising that they should have so long escaped the attention of marine zoologists."

A few of Cobb's (8) well-known words (as per reference 6) will illustrate his more modern and cosmopolitan outlook:

> "They (nematodes) occur in arid deserts and at the bottom of lakes and rivers, in the waters of hot springs and in polar seas where the temperature is constantly below the freezing point of fresh water. They are thawed out alive from antarctic ice ---. A thimbleful of mud from the bottom of river or ocean may contain hundreds of specimens. --- A lump of soil no larger than end of one's thumb may contain hundreds, even thousands of nematodes."

Cobb also refers to some of the unusual situations (6) in which nematodes have been found: beer mats in Germany; table vinegar; ear cockle of wheat; the human appendix.

The economic importance of nematodes, until recently, has been mainly restricted to the parasitic forms that affect the economic crops in agriculture. Hence, agronomists and plant-pathologists have been studying these organisms intensively in many areas of their interest. Besides these plant-parasitic forms, some of the zoo-parasitic forms have also been studied in relation to the diseases caused in economically important animals such as sheep, hogs, etc. However, the arrival of nematodes on the scene of sanitary engineering is relatively recent. The sanitary engineer's current interest in the free-living aquatic species resulted from the discovery of their presence in a city water supply in 1959. Chang et al. (9) first reported a water supply system in which these worms had been found. Articles such as, "How Pure is Your City Water?" (U. S. News and World Reports, Feb. 1960), dramatized the dangers of the presence of nematodes in city water supplies. Thus public attention has

been focussed on this subject, necessitating research work in this area. More attention is being given to nematodes in water supplies because of the many implications associated with their presence in drinking water. Besides being aesthetically objectionable, nematodes have been shown to be potential smugglers of pathogenic organisms from polluted waters into the treated waters through the treatment processes. Because of the fact that these organisms are largely produced in sewage treatment plants using aerobic processes, opinion has been voiced that the presence of these worms in drinking water could be an indication of pollution. As a result, the water supply industry is facing the problem of eliminating these worms from their water supplies. Considerable research effort is being directed towards an understanding of their occurrence, abundance, behavior and factors controlling nematodes in water so as to minimize their presence in drinking water.

LITERATURE REVIEW

As early as 1918, Cobb (10), who is considered as the father of nematology, discovered that slow sand filter beds were teeming with millions of nematodes. He also observed that the excretionary matter of these worms might be responsible for tastes and odors in water, as well as cause intestinal disturbances. Chang et al. (9) reported an investigation involving a city water supply system in which they found nematodes. The analyses were performed over a period of one month, June to July, 1956. Raw water was taken from the Ohio River and prechlorinated. Preliminary sedimentation for two days with or without alum-coagulation was followed by flocculation with ferric sulfate, sedimentation and filtration. Anhydrous ammonia was used to form chloramines with the residual chlorine. Activated carbon was used prior to flocculation, when it was found to be necessary. From the results of the

concentrations of the worms in raw water, chlorinated water, settled water, filter influent, filter effluent, and tap water, the authors arrived at the conclusion that the raw water was the source of the infestation of the water supply and that the predominating species of nematodes was Diplogaster nudicapitatus. The small number of worms in the water samples after final sedimentation indicated definitely that the worms were removed to a large extent in the flocculation and settling basins. However, some of the higher counts in some samples were attributed to the fact that a few gravid females might have given rise to a number of young or a large number of eggs hatched in the pipeline or sampling tap line. Lack of any significant difference in worm concentration between the filter influent and filter effluent indicated that the rapid sand filters were ineffective in removing the larval worms that did not settle out in the final settling tank due to their small size. It is of particular interest to note that the nematodes larvae remained viable for at least 100 minutes in a hypochlorite solution containing 1.8 to 2.4 ppm of residual chlorine at a pH of 8.3 to 8.4, at a temperature of 25°C. This amazing resistance explained the presence of living larvae in the distribution system after surviving the disinfection process. The worms in the distribution system may also be the result of reproduction in 'dead spots' in the pipelines. Even more resistant than the larvae, are the eggs, which, according to Kelly (11), could not be destroyed by 200 mg/ℓ of residual chlorine for a few days.

Chang et al. (12) surveyed 22 city water supplies for the presence of free-living nematodes, over a period of 11 months. Sixteen out of the 22 water supplies showed the presence of the nematodes. Of the 16, 15 supplies were obtained from rivers, and one used river water after impoundment. Of the six which did not contain nematodes, five were derived from lakes or impounded reservoirs and one from a river. The nematodes identified from the

samples of this survey belonged to the genera Monhystera, Aphelunchus, Rhabditis, Diplogaster, Cephalobus, Turbatrix, and Dorylaimus in decreasing numbers. The species of the genera Rhabditis, Diplogaster and Cephalobus were grown in pure culture. The cultural fluid, on benzene extraction, yielded a brownish, oily, gummy substance with a strong earthy, musty odor. One drop of this substance in a liter of water produced a distinct odor, supporting Cobb's observation regarding tastes and odors (10). However, whereas Cobb found these worms in slow sand filter beds, Chang et al. (12) observed that they were absent in rapid sand filter beds, although the authors indicated that nematodes might be present in the gravel portion of the filter bed. This is expected since there is only a short period of time between successive backwashings of a rapid sand filter and the worms would not have time to reproduce. Considering the incipient danger of the presence of the nematodes in water supplies, the authors suggested that a concentration of 10 worms per gallon or more should be sufficient cause of concern, and such supplies should be viewed with suspicion. An investigation should be made to trace their origin and remedial measures taken to eliminate them. This is particularly important if the species is Rhabditideae, because of their origin in the trickling filter beds of waste treatment plants (12). In this connection, a nematological survey conducted by Chang et al. (13) is of interest. They investigated the effluents from two waste treatment plants using trickling filters, three plants using primary settling alone, and one stabilization pond. Concentrations of worms as high as 2000 to 2500 per gallon were found in trickling filter plant effluents with a large number of the worms identified as belonging to the family Rhabditideae. The same species were found in the primary settled effluents, but the concentrations were lower, about 200 to 500 worms per gallon. The effluent from the stabilization pond contained only about 20 worms per gallon of the genus

Monhystera and two other genera. A bacterial analysis showed that there were 100 viable bacteria per nematode from the trickling filter plant effluent, about 75 viable bacteria per nematode from the primary settled effluent and only 30 viable bacteria per nematode from the stabilization pond effluent. Only 5 to 10 percent of these viable bacteria belonged to the coliform group, others being Psuedomonas, Proteus, Aerobactor, and Streptococcus. It is significant to note that Salmonella and Shigella and enteric viruses were not recovered from the nematode gut, even though Chang (14) had shown that the nematodes belonging to the genus Diplogaster can ingest pathogenic bacteria like Salmonella and Shigella and also the enteric viruses. It was also shown that the ingested organisms remained viable to the extent of 5 to 16 percent at the end of a 24-hour period with 0.1 to 1 percent being viable at the end of a 48-hour period. The most significant observation was that the ingested pathogenic organisms were protected by the carrier nematodes to the extent that they showed complete survival even when about 90 percent of the worms were immobilized by free chlorine. Another important factor worthy of note was that there were no viable bacteria or viruses in the excreta of the worms -- a fact sufficiently indicative of the complete digestion of the ingested pathogens by the worms.

Calaway (15) has recently described taxonomically and morphologically some of the predominant species found in the effluents of waste treatment plants. He also quoted Hausman (16), who in 1923 thought that nematodes were responsible for keeping the slime layer of trickling filter media porous by their burrowing action and also that these worms were responsible for the sloughing of the humus, thereby preventing clogging. Calaway pointed to the difference between the metabolic processes of bacteria and nematodes. According to Calaway, some of the food that bacteria ingest is released through

enzyme excretions, whereas the digestion products with nematodes are completely enclosed within the body and hence there is a smaller probability of food being released. The most common types of nematodes found belonged to the families Rhabditideae, Diplogasterideae, Mononchideae and Dorylaimideae. The last two families have been observed to be predators, preying upon rotifers, oligochetes and other nematodes, as compared to the other families which are only microphagus.

The work of Chaudhuri et al. (17) showed that the effluents from waste treatment plants were the major source of large populations of nematodes in streams. Urban and rural drainage contributions were considerably less. After the discharge of nematode-loaded effluents into the receiving streams, the authors traced the gradual disappearance of the nematodes from the flowing water and found the reduction could be attributed to various environmental factors such as temperature, dilution, and sedimentation.

Chaudhuri (18) has also reported a detailed study evaluating the potentiality of aerobic treatment plants as breeding sites for nematodes. He further substantiated that trickling filters are by far the major place of reproduction of nematodes, as compared with the aeration tanks of the activated sludge process. Laboratory studies were also conducted on two of the predominant species. The effect of such environmental factors as the area of culture flask, agitation, temperature and pH were evaluated. The important conclusions from these studies included the wide range of pH over which the nematodes can live and reproduce and the relatively short range of temperature for optimum growth.

The above summary briefly describes those aspects of free-living nematodes that the sanitary engineer is concerned with. It is evident that there is presently inadequate information to base any decision about the

possible sanitary significance of nematodes in water supplies. This led to the fairly logical stand taken by the American Water Works Association (19), which is summarized as follows in the words of Calaway (15):

> "Since the presence of nematodes in raw and finished water has been known for a number of years, and there are no known cases in which the relation of their presence to diseases has been traced, their occurrence in water, while not desired, need not cause excessive alarm."

Nevertheless, because of the obvious importance to the water supply industry such an attitude should not hinder further research for a better understanding of these worms.

SCOPE OF PRESENT STUDY

Free-living nematodes have been recognized as essentially aquatic organisms -- either living in water or in films of moisture on soils. There is extensive evidence in the literature as to their prevalence in water and soil. This aquatic nature of nematodes is an important aspect in any study involving ecology, because nematodes of soil origin may end up in drainage courses due to the flushing action by overland runoff. Once in the stream, the fate of these nematodes is determined by one or more of the various environmental factors such as velocity of flow, temperature, chemical composition of water, availability of food, pH, dissolved gases like oxygen, and also on the other biotic communities present. As reviewed earlier, considerable effort has been directed towards the nematodes found in water. However, there are no reported studies on the nematodes that have long been known to be present in large numbers in the bottom mud of streams. The fact that the nematodes found in the bottom mud have a definite effect on the occurrence of nematodes in the water above cannot be overemphasized. Therefore, the present study was undertaken to investigate some of the factors involved in the

occurrence, abundance and behavior of the nematodes in bottom mud -- or the benthos. In this ecological study of the stream benthos, an attempt was made to delineate the relationship of, (a) the concentration of nematodes in the benthos with the discharge of nematodes in the effluent of a waste treatment plant, (b) the concentration of nematodes with respect to the physical and chemical properties of the bed material, and (c) the spacial distribution of nematodes in the stream bed. In addition, controlled laboratory studies were made on the growth characteristics of mixed cultures of nematodes on a heterogeneous media.

Due to the convenience of the waste treatment plant of the Urbana-Champaign Sanitary District, the Boneyard-Saline Branch Drainage system was chosen as the site of study. This stream system was studied by Chaudhuri et al. (17) in their studies on surface waters.

II. TECHNIQUES OF BENTHIC SAMPLING AND ANALYSIS

GENERAL CONSIDERATIONS

Any sampling procedure for biological analyses is quite different from sampling for chemical analyses. The chemical constituents are usually dissolved and are, therefore, uniformly distributed, whereas the living organisms are generally not uniformly distributed. Again in contrast to sampling of water for floral and faunal analysis, sampling of bottom material or benthos is still more complex. This complexity is obvious because of the wide variety of bottom material such as rocks, stones, gravel, sand and silt which occur in varying proportions in the benthos besides the conditions of the water, such as lotic or lentic, or deep or shallow. In addition to these, any sampling procedure will have to conform to the nature and size of the organism or organisms under investigation. In this respect, the micro- and macroorganisms would need to be considered separately. A chemical analysis is almost always done on a quantitative basis, but a similar quantitative biological analysis is beset with many problems which necessitates the reporting of results on a qualitative basis, with some qualifications in respect to the relative abundance of different species. The problem of quantitative biological analysis of benthic samples is further complicated by the fact that isolation of the organisms under study from the debris and ooze is necessary before identification and enumeration can be done. The accuracy of the quantitative results therefore depends largely on the efficacy of the method employed for isolation of the organisms. In any particular investigation, all these considerations are preceded by those of collection of the samples, which in turn involves other factors. In brief, the following factors are to be considered in a benthic study:

I. Collection of Samples

1. Selection of sampling sites

2. Sampling devices

3. Sampling frequency

II. Isolation of fauna and their enumeration

COLLECTION OF SAMPLES

Sampling Site. Because of the proximity of the Boneyard-Saline Branch drainage system to the Sanitary Engineering Laboratory and also because of the earlier work done on water samples from this stream system (17) the present study of benthic samples was taken up on the same stream system as shown in Figure 1. Boneyard Creek carries the urban discharge from the twin cities of Champaign and Urbana. The Saline Branch receives entirely rural drainage until it enters Urbana when it is joined by the Boneyard. The Urbana-Champaign Sanitary District waste treatment plant discharges its effluent about 400 yards below the confluence of the two streams. The Saline Branch drainage ditch joins the Salt Fork Drainage Ditch about 10 miles downstream from the effluent outfall of the waste treatment plant.

The sampling stations selected are marked A through F on Figure 1. Stations A is on the Boneyard creek, and Station B is on the Saline Branch below the confluence with the Boneyard but above the outfall of the effluents from the waste treatment plant. Stations C through F are on the Saline Branch downstream from the waste treatment plant. The two stations upstream of the waste treatment plant were selected to study the effects of the effluent discharge on conditions above and below the outfall, and the four stations below the outfall were chosen to study the fate of nematodes downstream. All samples collected at any one station although obtained at different times were within a stretch of about 15 feet.

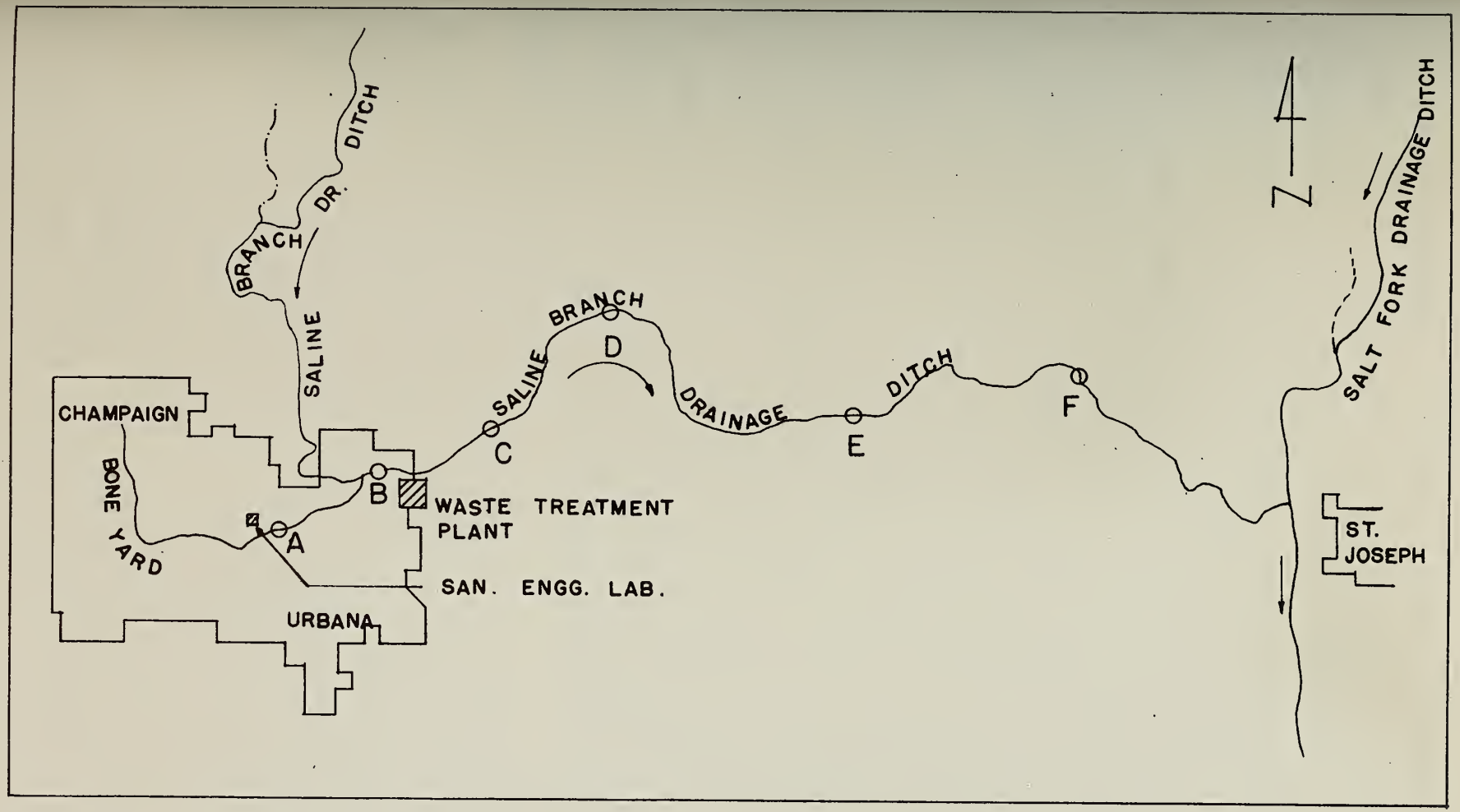


FIGURE 1. BONEYARD - SALINE BRANCH STREAM SYSTEM

Sampling Devices. As mentioned earlier in this chapter, a number of problems arise in quantitative benthic sampling for biological analysis. Sampling procedures have been used to suit local situations in individual cases, and involve different principles. One of the most common methods of benthic sampling is with some type of dredge. Various sizes and types of dredges have been described in the literature for different conditions (20). The Ekman Dredge for soft-bottom deposits, and the Peterson Dredge for hard-bottom deposits are just two examples. Another method of taking a sample consists of drilling a core out of the bottom sediments by means of a core-sampler. The modified Wilding Sampler and the Young's Hollow Square Sampler are examples of this kind. Other samplers designed to obtain samples from the ooze at the interface of bottom material and water are also available. In still other cases, ordinary scoops have been used to obtain bottom samples. Using the above principles, various modifications have been incorporated to produce sampling devices best suited to a particular situation. In fact, it is impossible to have one sampler suitable for all types of habitats, therefore, the number of samplers used is nearly proportional to the number of individual investigations (21).

The stream bottom of the Boneyard-Saline Branch system was predominately sandy, with varying amounts of pebbles or gravel. The stretch of the river between the outfall and Station C is rocky, and that was the reason for not establishing a station in this reach. The Ekman dredge was found to be quite suitable for obtaining a bottom sample and was used initially while a method of isolation of the organisms from the samples was being perfected. When it became imperative to obtain an undisturbed sample from the stream bed in order to make measurements of the oxidation-reduction potential, the Ekman dredge could no longer be used. Since the oxidation reduction potential is

very sensitive to oxygen concentration, a disturbed sample would be of no use for this purpose. Therefore some manner of obtaining an undisturbed sample had to be adopted. In this particular case, advantage was taken of the fact that the stream under study was shallow, with water depths rarely exceeding 2 feet. Under these circumstances, a simple scoop appeared to be quite suitable. On a trial basis, a scoop was made out of a one-gallon motor oil can by cutting it longitudinally so that each end formed a segment of a circle with a chord length of 6.5 inches and a maximum depth of the scoop of 3 inches (Figure 2). The length of the scoop was 7.5 inches. During sampling, the person doing the sampling faces upstream, holding the scoop with its longitudinal axis parallel to the flow. Then lowering it, the scoop is driven slowly and steadily forward with a circular motion till the scoop faces almost vertically with a portion of the bed material now inside the scoop. The scoop is driven to about a depth of two inches, leaving a depth of about one inch of water above. The position of the scoop may be adjusted slightly in situ by very gently moving the scoop in the bed itself. After the sample is obtained, the scoop is raised slowly from the bed, taking great care to keep the sample undisturbed. This operation, when carried out with skill, yielded very good samples, as can be observed from the surface of the sample in the scoop. A little practice with the scoop will certainly help in obtaining better samples. Particular care was taken to avoid the effects of the velocity of flow of water above while the scoop was being lifted from the bottom. If the trial was not successful, a second trial was made nearby. The scoop was found to be quite suitable for the sampling in this investigation and was used extensively.

Sampling Frequency. Samples were collected every 10 to 12 days as dictated by the time needed for the analysis of samples collected. Samples were collected from any two stations on one day, twice a week, spread over six months from June to November, 1963.

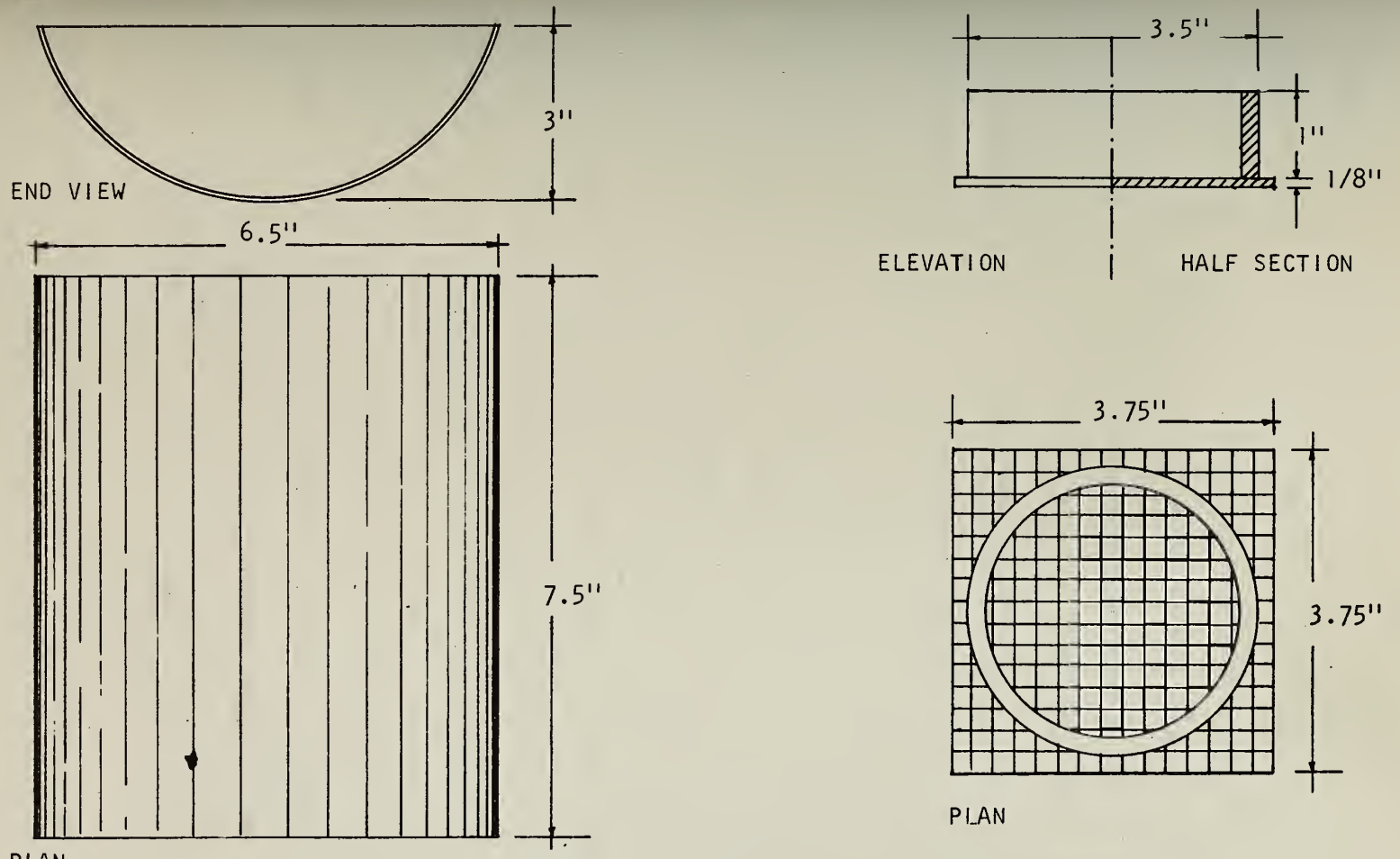


FIGURE 2. SAMPLING SCOOP AND COUNTING DISH

ISOLATION AND ENUMERATION

After obtaining a satisfactory sample, the next thing is to isolate the organisms of interest -- in this case, the nematodes. A quantitative biological analysis of the bottom sample is very complicated because of the inherent difficulties faced in separating the organisms from the large mass of bottom material including dead organic matter, plants, roots, leaves, and other forms of life. Once the organisms have been separated, enumeration can then be done. Various methods have been used for the isolation of the organisms in benthic samples. The most thorough, but also the most time-consuming, procedure for removing animals in benthic samples is by hand-sorting. Besides being very tedious, this method is more suited for macroorganisms than for microorganisms. Seiving is another method used to isolate the animals from the bottom material. Here the organisms as well as other materials of sizes within a given range will be separated. Also, this method is again more suited for the macroorganisms than for microorganisms. Elutriation offers some promise of successful separation. Lauff et al. (22) have described a bubbler in which a large sample is first vigorously agitated in a column of water using compressed air, the agitation being controlled to float only the organisms of interest, allowing the heavier debris to stay at the bottom of the column. The supernatant can then be drawn off. In a modification of this bubbler, after complete agitation, the column of water may be allowed to stand for just the time needed for settling of the heavier portion, and the supernatant drawn off at the end of this period, as compared to the immediate removal of the supernatant. The supernatant so drawn off may then be passed through a seive of required size to concentrate the organisms. However, the method appears to be unrefined, though sound in principle. The Baermann funnel (4) method is still another method for the separation of organisms -- especially the

microorganisms. The organisms are filtered through a membrane, cheesecloth, linen cloth, bolting cloth or even facial tissue placed in a funnel and into a rubber tubing connected to the stem of the funnel full of water, from which organisms can be drawn off. The gravity and motility of the organisms are the important factors in the success of this method. Another disadvantage is the long period of time needed for complete settling of the organisms in the tubing. For this reason the efficiency of the removal of organisms has often been questioned.

The flotation method appears to be the most suitable. The principle involved in this method is that an organism of a given specific gravity will float when placed in a solution of sensibly higher specific gravity. The specific gravity of most of the organisms and other detritus material in the benthic sample (except a few plants and algae) is generally about 1.12. The specific gravity of most of the invertebrates, except some snails and clams, is less than 1.12. Therefore, when a sample of benthic material is placed in a solution of specific gravity 1.10, most of the invertebrate organisms will float to the surface, while most of the organic detrital material will sink to the bottom. Anderson (23) described a method for sorting bottom fauna by the flotation principle using a sugar solution. An important factor to be considered in the flotation technique using sugar solution is the length of time the organisms remain in contact with the sugar solution because it has an effect on the recovery of the organisms from the sample. Animals tend to shrink due to a fluid loss in any hypertonic solution and, therefore, increase in specific gravity. In addition, they become morphologically distorted. When the specific gravity becomes greater than that of the solution, the organisms sink, unless retained by surface tension. Further, the hypertonicity or the osmotic pressure of different solutions at a given specific gravity is

dependent on the molecular weight of the solute and the number of the ions or molecules in the solution. In this respect, flotation time can be longer in sugar solution than in a solution of inorganic salts. For animals exposed to the hypertonic effects below the lethal level, the original specific gravity can be reconstituted by soaking in water for ten to twenty minutes. Even with this limitation on flotation time, the method is very useful. However, Caveness et al. (24) have described a modification in the flotation principle in which centrifugation is used to enhance the flotation thereby reducing the time of exposure. The method has been used by these authors for isolation of nematodes and their eggs from plant tissues and soils. A sugar solution of specific gravity 1.10 to 1.35 was used with a flotation time of 3 to 9 minutes, the rate of centrifugation being 4,800 rpm. The specific gravity of the sugar solution used was satisfactory, because the specific gravity of nematodes has been found to be from 1.05 to 1.06. The method as used by Caveness et al. was as follows (24). The samples to be treated were placed in cellulose nitrate centrifuge tubes of 60 ml capacity and approximately 30 ml of water were added to each. The tubes were then centrifuged simultaneously for five minutes, in a SerVall centrifuge. Timing began after a maximum of 4,800 rpm had been reached. The resulting supernatant liquid was poured off with one smooth operation without disturbing the material at the bottom of the tube. This spinning of the sample eliminated the material lighter than water and was repeated as needed. Nematodes were recovered from the residue in the tubes by addition of a sugar water syrup having a specific gravity of 1.18 and centrifuging as before. After spinning, the syrup containing the nematodes was immediately diluted by decanting the supernatant into 500 ml of tap water, to avoid any damage to the nematodes. The excess water was decanted after 20 minutes, when the nematodes had settled. The water and nematodes were

poured in a liter graduate and the volume made up to 250 ml. After shaking the graduate, a 10-ml aliquot was taken immediately and allowed to settle on a Syracuse dish marked for counting. The procedure may be repeated if the recovery of nematodes has not been complete in the first flotation. These authors also ran the Baermann funnel method on the same samples for the purposes of comparison. The results led the authors to conclude that the recovery by the flotation method was more complete than in the Baermann funnel method. The flotation method was also found to be suitable for rapid processing of small quantities of samples. This method has the greatest advantage of reducing the objectionable extraneous matter from the sample.

Considering the advantages of the flotation technique, it was decided to try this technique in the present investigation. However, some modification of the centrifugal-flotation technique appeared to be necessary in view of the particular needs of the present study. The five principal factors in the modification are:

1. Specific gravity of sugar solution
2. Preparation of the sample after bringing it from field
3. Amount of sample to be used in flotation or size of aliquot
4. Time of flotation
5. Method of counting

Individual consideration was given to each of the five factors above which make up the complete procedure. As mentioned earlier, nematodes have a specific gravity of 1.05 to 1.06. Any solution of sugar of specific gravity appreciably higher than this should be sufficient to float the nematodes. Using a solution of considerably higher specific gravity would involve the harmful effects of hypertonicity. Therefore, the lowest specific gravity that could be used without impairing the efficiency of flotation, was 1.10,

which is the lower limit used by Caveness et al. (24). All flotations were carried out using a sugar solution of specific gravity 1.10.

As mentioned earlier, the stream bed at the sampling stations consisted mostly of sand with varying amounts of gravel. A sample, as collected in the scoop, was therefore a heterogeneous mixture of sand and gravel. A certain amount of sample-preparation was necessary before a representative aliquot could be obtained. As a first step, the bottom sample was allowed to drain by tilting the scoop and allowing the water to drip away very gradually. Once the sample was drained, about 50 gms from the center portion of the scoop was thoroughly but very gently mixed omitting the larger gravel. A very gentle mixing was very important because otherwise there was danger of crushing the delicate worms. The mixing was done in the scoop for at least three minutes using a spatula. An aliquot was taken from this mixture for centrifugal-flotation. Since the aliquot taken was small, gravel or sand grains larger than 2 mm were eliminated. By measuring the proportion of the gravel larger than 2 mm in the whole sample, the results of flotation were extrapolated to correspond to the whole sample collected. Also, by measuring the area of sampling and the total weight of sample collected, the nematode count could then be expressed in terms of the area, from the known concentration per unit weight.

After preparation of the sample, as described above, a suitable aliquot was taken. It should be recognized that, in spite of the thorough gentle mixing of the sample, a small aliquot may not be truly representative of the whole sample. At the same time, too large an aliquot would also be undesirable because the large mass of bottom material may interfere with the complete recovery of nematodes by enmeshing them in the debris. In order to determine the optimum size of an aliquot, a series of tests were made by

taking aliquots of different sizes, 1.0, 5.0, 10 and 20 gms, from the same sample and the results of the centrifugal flotation for a three-minute spinning were compared. Generally it was found that 1.0 gm sample yielded the lowest concentration of nematodes per gram, thereby indicating that it was too small an aliquot to be considered representative. The concentration of nematodes per gram from the 5.0 and 10 gram aliquots were remarkably close to each other, and also the maximum found. The nematode concentration per gram from the 20 gram aliquot was again smaller than that of 5.0 or 10 gram aliquots, but more than that of 1.0 gram aliquot. From these results, it was concluded that a 5.0 or 10 gram aliquot would be an acceptable size of sample for flotation, and further that, in view of the close agreement of results of either aliquot, the method of recovery was also very satisfactory.

It has already been mentioned that time of flotation is a very important factor because of the adverse effects of hypertonicity on the nematodes. A three-minute flotation would actually expose the nematodes to sugar solution for about ten minutes because of the time required for the various manipulations to be carried out. Even though there was no evidence of a detrimental effect with a ten-minute exposure, an attempt was made to reduce the time of flotation to one minute, and thus reduce the possibility of any harmful effect. However, a comparison of the results of the one-minute and three-minute flotations showed that, from identical aliquots, one-minute flotation yielded fewer nematodes than the three-minute flotation. It was therefore concluded that a three-minute flotation time was necessary for best recovery, and was used in all the analyses.

The supernatant containing the nematodes was then carefully decanted into a liter of water in a beaker. It should be noted here that more than one flotation was necessary when the total number of nematodes in the sample was

more than about 100. In such a case, the centrifuge tube was filled with about 40 ml of tap water after the supernatant had been decanted from the first flotation. The tube was then allowed to stand for about 30 minutes to allow the nematodes to recover from the hypertonic effects of the sugar solution. The tube was then centrifuged as before and the supernatant water decanted into the beaker containing the first flotation supernatant. Sugar solution was then added to the tubes for the second flotation, and the residual nematodes recovered in the same manner as before. Once the sugar solution had been diluted in the beaker, the nematodes were allowed to regain their shape, if distorted, for about 30 minutes. At the end of this period, instead of taking an aliquot out and counting in a Syracuse dish as was done by Caveness et al. (24), a different procedure was followed for enumeration. The entire contents of the beaker containing the nematodes were filtered through a 5 μ membrane filter, the rinsings of the beakers also being added. The filter containing the nematodes was then placed in a test tube, and a two percent Eosin-Y dye solution added in sufficient quantity to submerge the filter paper in the test tube. The dye solution was used for the staining of the nematodes as per the staining technique developed by Chaudhuri et al. (25). It has been found to be extremely useful for a quick enumeration of nematodes in the counting dishes, because of the ease with which stained nematodes can be identified. A period of about 24 hours was allowed for the uptake of the dye by the nematodes. At the end of this period, the contents of the test tube was filtered through another 5 μ membrane filter. The nematodes and other material retained on the original filter were carefully removed by scraping and washed onto a second filter, all rinsings of the test tube and first filter again being added onto the second. This second filtration enables the isolation of the stained nematodes from the body of the staining solution. The second filter

was then carefully washed by scraping off into a counting dish. The counting dish had been specially made with a circular well with vertical walls with a squared bottom plate -- Figure 2. The washing of the second filter was done with a minimum amount of water to keep the depth of water in the counting dish low so as to avoid difficulty in focussing during counting. The counting dish was then placed under a dissecting microscope and each square of the counting dish was brought under the microscope under a magnification of 24x. By carefully moving the counting dish, every square of the counting dish was brought in focus and the nematodes in each square counted.

The above discussion of the procedure for isolation of nematodes from the bottom samples obtained with the scoop in the field is summarized as follows:

1. Drain the sample and thoroughly mix about 50 grams of the sample at the center of the scoop, leaving particles larger than 2 mm. This must be done gently to avoid destruction of the nematodes.

2. Centrifuge a 5-gram aliquot of this mixture in a tube with 40 ml of sugar solution of specific gravity 1.10, for three minutes at 4,800 rpm.

3. Dilute the sugar solution by carefully decanting the supernatant into a beaker containing a liter of water.

4. Add the supernatants of any subsequent flotations to the beaker.

5. After 30 minutes, filter the contents of the beaker through a membrane filter.

6. Place the membrane filter in a test tube, and add sufficient two percent Eosin-Y dye solution to immerse the filter.

7. After one day, wash the deposit on the filter and the contents of the test tube onto another membrane filter.

8. Remove and rinse the second filter with a minimum amount of water into a counting dish.

9. Count the nematodes in the counting dish using a dissecting microscope at 24x.

III. BENTHIC ECOLOGY OF NEMATODES -- RESULTS AND DISCUSSION

Ever since Bastian (7) and Cobb (8, 10) made known the universal presence of nematodes, a great many workers have found and reported the presence of these organisms. With the recognition of their possible signifucance in Sanitary Engineering in recent years, several studies on the free-living, fresh-water nematodes associated with water supply and waste treatment processes have been reported. Studies have also been reported about their occurrence in surface waters (26). However, no specific studies have yet appeared in the literature regarding the occurrence and abundance of nematodes in the benthos. It was the object of the investigation reported here to investigate some of the ecological factors relating to the nematodes in the stream benthos.

SOURCES OF NEMATODES IN THE BENTHOS

The stream system under investigation is shown in Figure 1, along with the sampling Stations A through F. This portion of the study was made to observe the relative abundance of nematodes in different sections of the stream system, and also to evaluate the effect of the waste treatment plant effluent on their occurrence. Water and benthic samples were obtained from the six stations (Figure 1). The nematode count of the bottom samples was done using the centrifugal-flotation technique as outlined in Chapter II and results expressed as nos/square inch. The nematode count of the water sample was done by filtering an aliquot of 500 ml through a membrane filter and counting after staining, with the results expressed as nos/gallon. The pH and temperature measurements were made immediately after obtaining the samples in the field. Also the oxidation-reduction potentials of the supernatant

water and of the bottom material at depths of 0.5 cm and 2.0 cm in the field were measured and expressed as E_7 values after correction for pH. A dissolved oxygen sample was also taken of the water, and fixed in the field. Other tests done on the 103°C-dried sample included the chemical oxygen demand, total nitrogen, and effective size and uniformity coefficient of the bottom material. The results of all analyses are presented in Tables I, II, III and IV in the Appendix. This sampling program was carried out during the period of June 1963 to August 1963, when the water temperatures ranged from 22° to 30°C. Figure 3 shows the average nematode concentration in the benthos for this period. The nematode concentration in the water samples for this period is also shown in the figure. The average surface velocity of the water as determined with a float for each of the downstream Stations C through F, and the dissolved oxygen concentration averaged for the entire period, are also shown.

Chaudhuri et al. (17) have shown that for the water samples obtained from the stream system, the nematode concentrations are higher in Boneyard Creek carrying urban drainage than those of the Saline Branch carrying rural drainage. The high concentrations occurring immediately below the outfall of the waste treatment plant effluent, follow a successively decreasing pattern as was established for the period December 1962 to April 1963. The same type of results were also obtained in this study for the samples obtained for the period June through November, 1963, as seen in Figure 3. With respect to the bottom concentrations of nematodes in Boneyard Creek, it is seen that Station A on the Boneyard has a higher concentration than that of Station B, which is on the Saline Branch below the confluence of the two streams. This diluting effect is attributed to the discharge from the Saline Branch which has a lower concentration of nematodes than Boneyard Creek. However, the

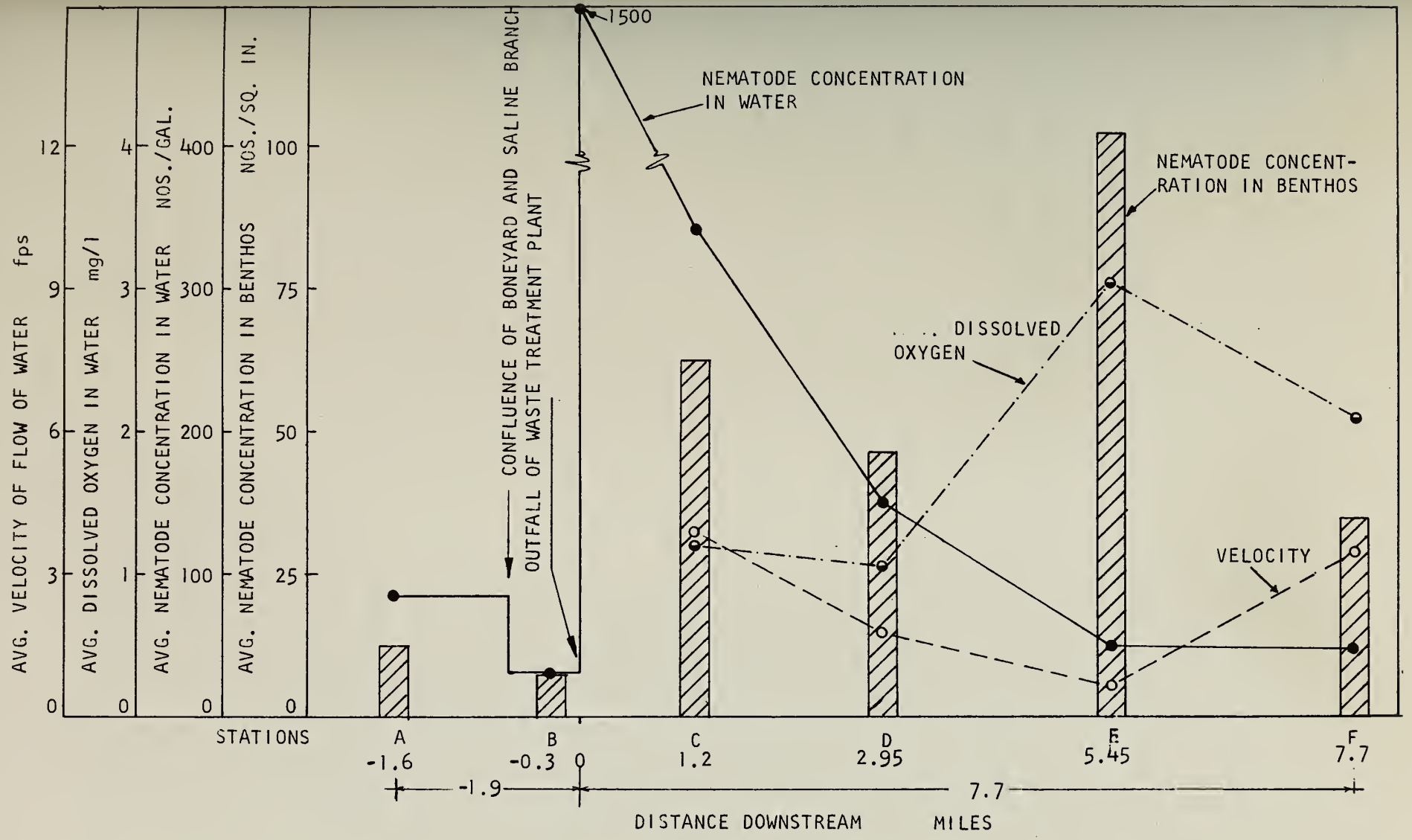


FIGURE 3 AVERAGE NEMATODE CONCENTRATION IN WATER AND BENTHOS AT STATIONS A THROUGH F FOR THE PERIOD JUNE TO AUGUST 1963

effluent from the waste treatment plant increased the nematode concentration in the stream bed below the outfall tremendously. This significant increase in bottom concentration is attributed to the large number of nematodes discharged in the effluent which continuously seed the stream. Also the treatment plant effluent provides an additional source of food in the form of bacteria. It is interesting to note that the concentrations of nematodes in the benthos do not follow the decreasing pattern observed for the water. As a matter of fact, the average nematode concentration for Station E was considerably higher than the other stations. This fact indicates that there was no correlation between the nematode concentration in the water and the nematode concentration in the bed material, in which case there must be some other factor that is responsible for this anamalous pattern. A look at Table II shows that the bottom material was almost of the same quality as regards the effective size and uniformity coefficients. Therefore the physical properties of the substrate in terms of effective size and uniformity coefficient are ruled out of being responsible, as far as this investigation is concerned. The only reasonable factors appear to be the average velocity of flow and the dissolved oxygen concentration. Figure 3 shows that the velocity of flow was lowest at Station E, where the maximum concentration of nematodes occurred in the stream bed. This strongly suggests that the absence of water currents is a major factor either in causing a more rapid growth of nematodes in the benthos or in preventing the erosion of bed-material, thus allowing the nematodes to remain in the benthos instead of being flushed away. Also the absence of currents enhanced the settling of nematodes from the water on the bottom. Another factor contributing to the high concentration at Station E may be the dissolved oxygen supersaturation. During the entire period of sampling, large algal blooms and water plants were at this station, which was partly a

result of the sluggish velocities, and accordingly, the water samples were generally supersaturated with dissolved oxygen. This might have kept oxygen levels in the benthos at a higher level. However, dissolved oxygen at other stations was rarely below 2 mg/ℓ and hence never was limiting, in view of the fact that nematodes have been known to be present in nearly anaerobic conditions (27).

During the above period, namely, June to August, 1963, the water temperature varied from 22° to 30°C, with an average temperature of 25.1°C. In order to ascertain the effects of seasonal variations in temperature, a similar sampling program was undertaken during October and November, 1963. During this period the temperature range was observed to be from 15° to 24°C, with an average temperature of 18.5°C. For the water and benthic samples obtained during this period, the nematode counts were made, along with the usual pH, temperature and dissolved oxygen measurements. The other physical and chemical analyses were omitted. Results of this sampling are presented in Table V in the Appendix. The average values of dissolved oxygen and nematode concentration per square inch are also given. Figure 4 is drawn to make a comparison of the data of the two periods, June through August, 1963, and October through November, 1963. The top half of the Figure 4 shows the nematode concentrations in water and bottom material during June through August, 1963. The bottom half of the figure shows the nematode concentrations in water and benthos during the period October through November, 1963. It can be seen from the figure that the same general pattern of nematode concentrations in water and bottom was observed during both periods, with the difference that the average concentrations generally were all considerably higher during October and November, 1963. It should be noted here that the average concentration of nematodes in the stream water had also increased due to seasonal

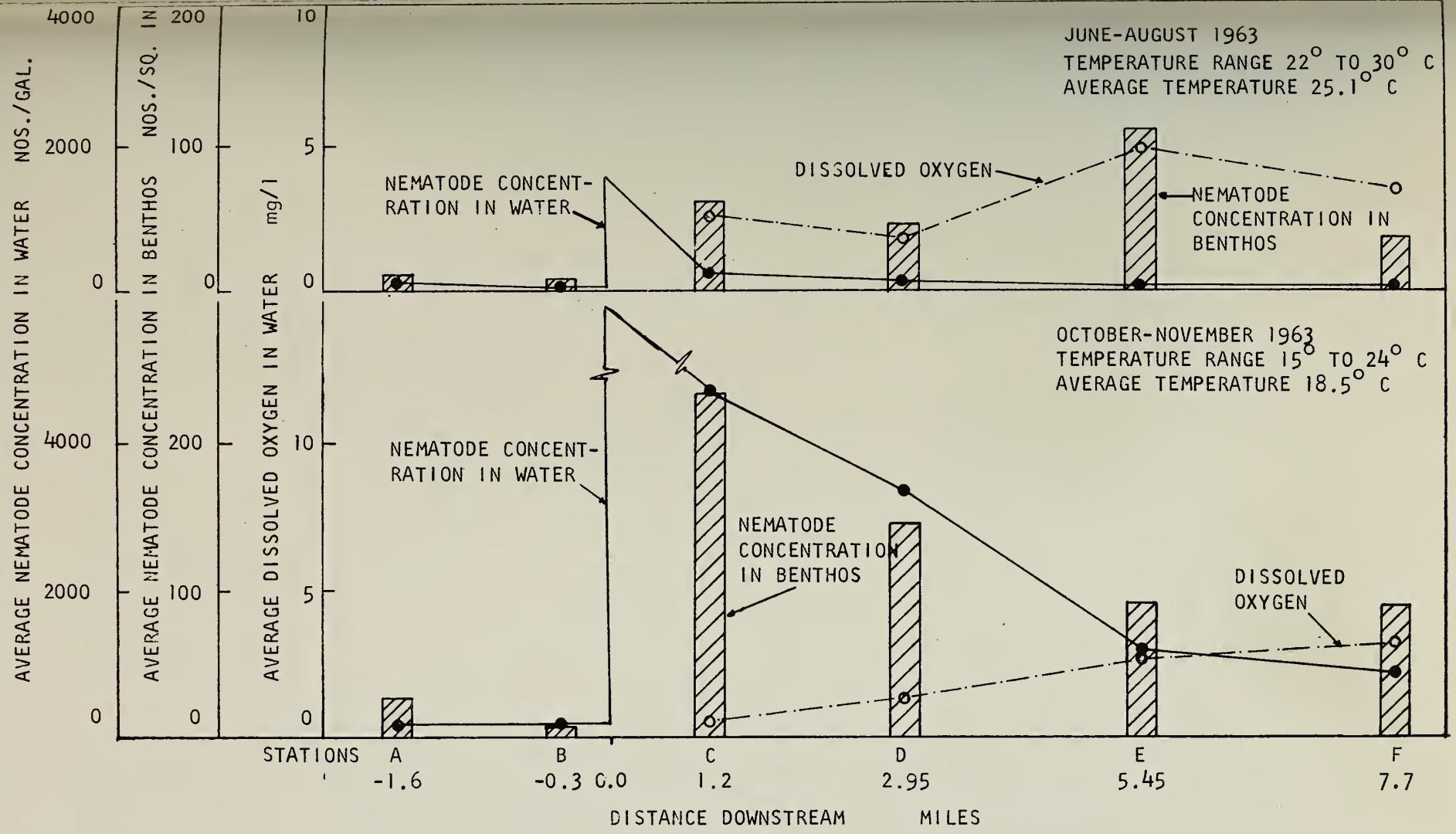


FIGURE 4 COMPARISON OF NEMATODE CONCENTRATIONS IN WATER AND BENTHOS AT STATIONS A THROUGH F FOR THE PERIODS JUNE - AUGUST 1963 AND OCTOBER - NOVEMBER 1963

variations. But the bottom concentration did not increase in the same proportion. As a matter of fact, in comparing the data for the two periods, the average concentration in stream water downstream increased 17 times, whereas the average concentration in bottom material downstream increased only about twice. This increase was probably due to the more favorable temperatures rather than any other reason. It is interesting to note that for the period October-November, 1963, Station E had not shown any unusually high concentration as was observed for the period June-August, 1963. One significant difference is the absence of supersaturation of dissolved oxygen. Unfortunately, no velocity measurements were made to substantiate the earlier hypothesis.

EFFECT OF ENVIRONMENTAL FACTORS ON NEMATODE CONCENTRATION

The above discussion is based on the average concentrations of nematodes observed at each station, with the aim of tracing the origin of the nematodes in the stream bed. Temperature as an environmental factor has already been considered in discussing the relative abundance of nematodes in the stream bed. Additional comments on the effects of temperature changes will be discussed in the next section. In this section, such environmental factors as currents and high flows, chemical properties like Chemical Oxygen Demand (COD), total nitrogen, pH and physical properties of the bottom material such as effective size and uniformity coefficient will be considered.

Currents and variations in flow are important ecological factors for any organism in a stream, in the water and in the benthos. The organisms in the benthos are continuously exposed to a changing stream of water, flowing at different velocities. When there is a high or flood flow in the stream, the effects are more marked than usual. Within limits of daily normal variations of flow, the littoral zone is less affected than the mid-stream zone.

Both are affected by high flows depending on the degree of inundation. With a view of studying the effects of variations of flow, including high and flood flows, graphs are presented showing the flows in the stream and the nematode concentrations in the benthos for different sections of the stream system. Figures 5 through 10 show the different stations of the stream system. The daily peak flows only are plotted because of their importance. The daily peak flows in Boneyard Creek were obtained from the United States Geological Survey Office in Champaign. The combined daily peak flow in the Saline Branch, after the confluence with the Boneyard, was obtained from a self-recording gauging station located just above the outfall of the waste treatment plant. The peak flow in the Saline Branch below the outfall is calculated by adding the maximum effluent rate of the waste treatment plant to the peak flow at the recording gauge. It was a matter of misfortune that the self-recording station was not operative after October 25, 1963. For the period after this date, the peak flow in the Saline Branch was computed from the average dry weather flow for the previous 50 days together with an assumed flood flow from the daily precipitation data obtained from the local United States Geological Survey Office. The flood flows from the precipitation data have been assumed on the basis of previously recorded flood flows from comparable precipitation in the area. This is admittedly approximate, but it is felt that such data would at least indicate the periods of high flows. To distinguish this portion of the graph drawn on assumed values, the curve is drawn by broken lines.

Figures 5 and 6 represent the conditions for Stations C through F, plotted for the period June to August, 1963. Prior to the peak flow of July 5, 1963, the concentrations of nematodes in the stream bed at Stations C through F, were considerable. However, due to the erosive action of the high flow of July 5, the nematode concentrations were reduced. The later high

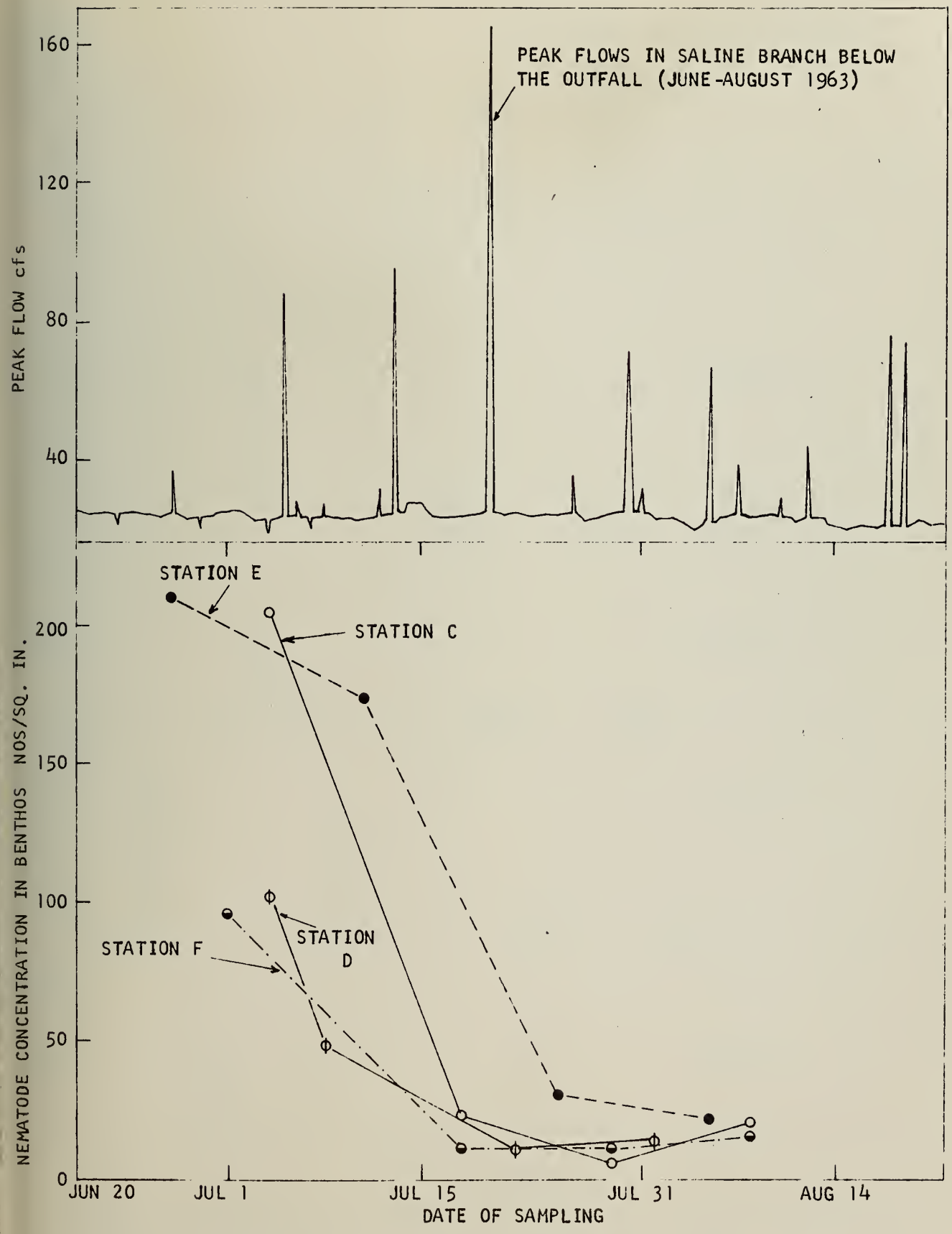


FIGURE 5 VARIATIONS OF NEMATODE CONCENTRATION IN BENTHOS WITH PEAK FLOWS -- STATIONS C THROUGH F (JUNE-AUGUST 1963)

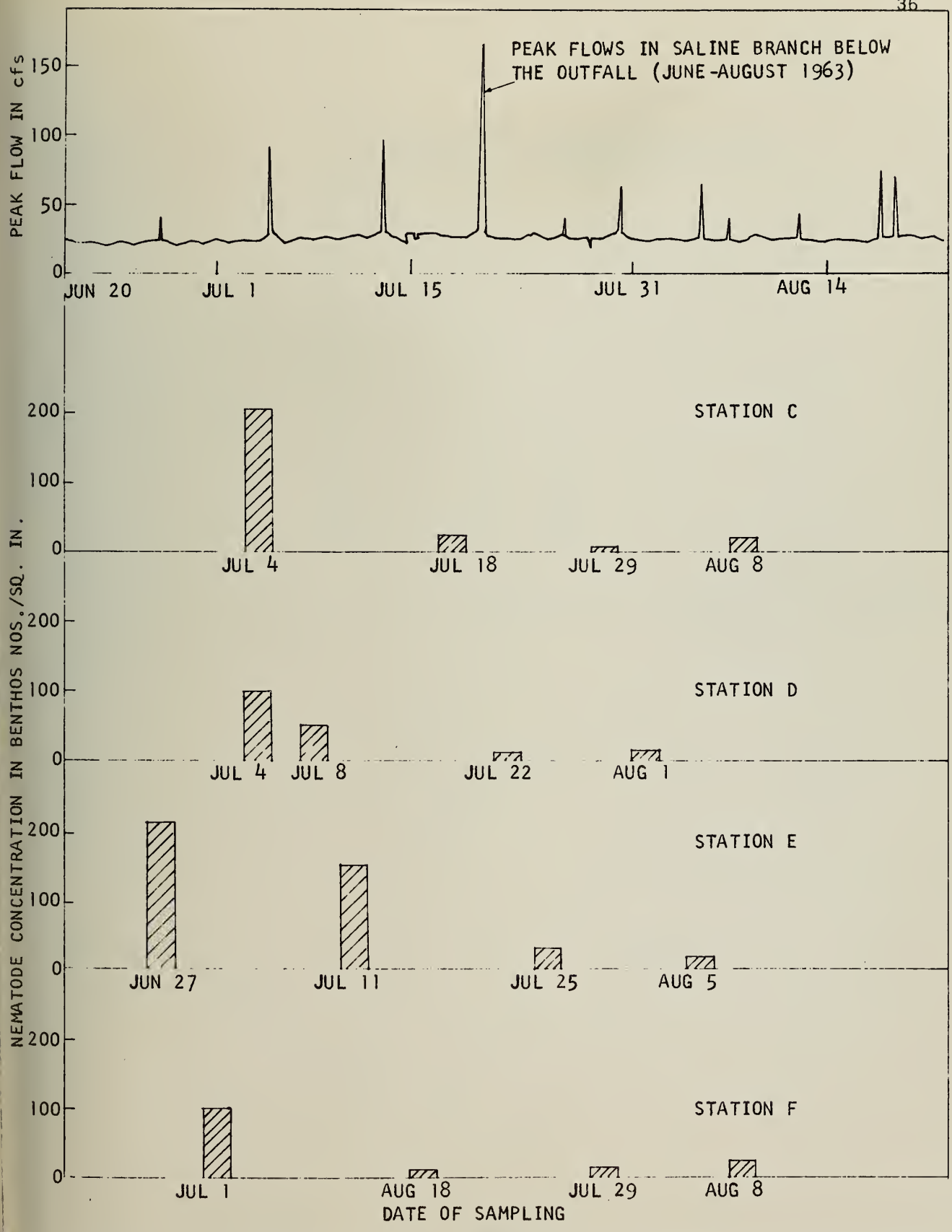


FIGURE 6 VARIATIONS OF NEMATODE CONCENTRATION IN BENTHOS WITH PEAK FLOWS AT EACH STATION C THROUGH F (JUNE-AUGUST 1963)

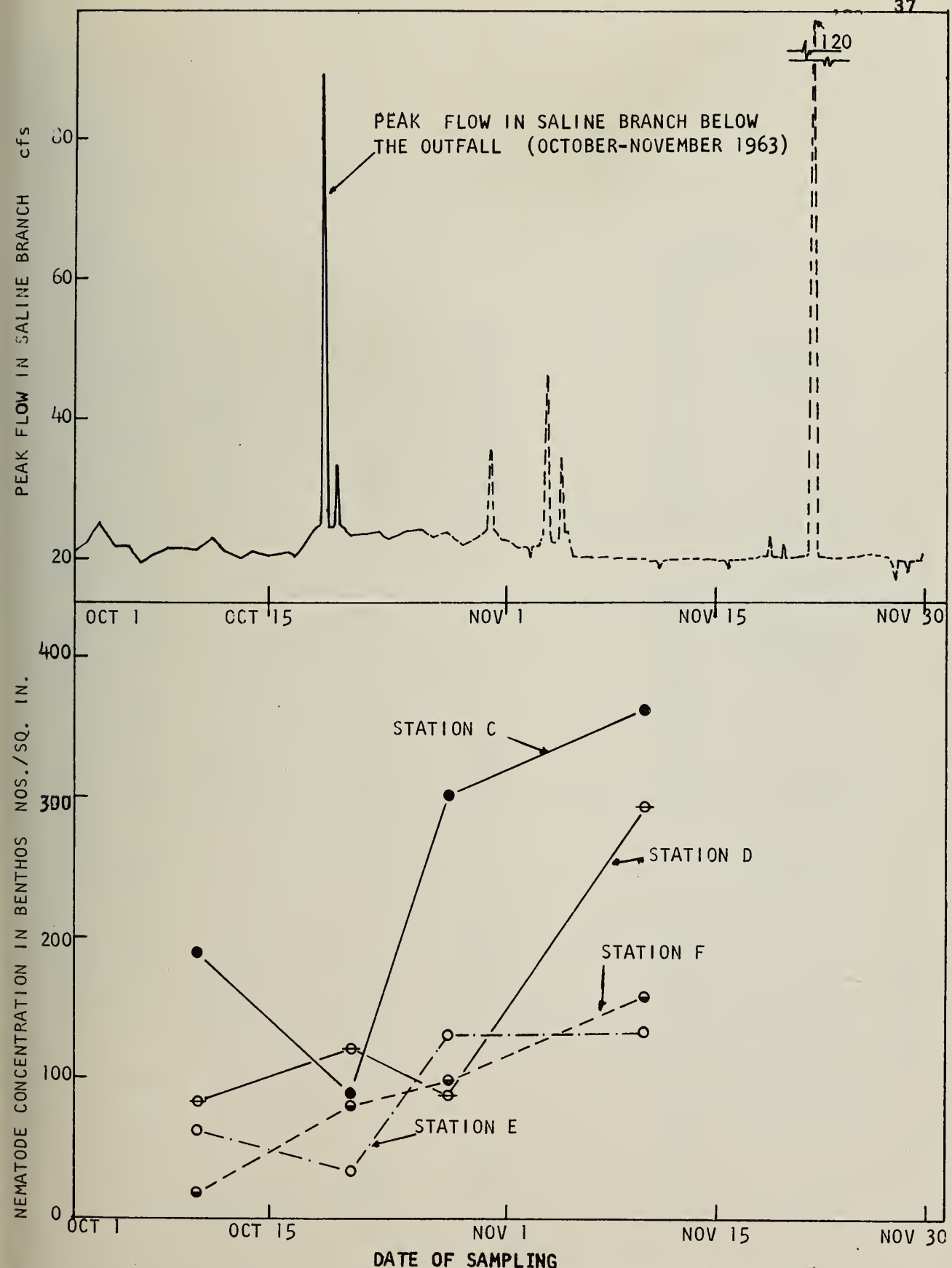


FIGURE 7 VARIATIONS OF NEMATODE CONCENTRATION IN BENTHOS WITH PEAK FLOWS - STATIONS C THROUGH F (OCT -NOV 1963)

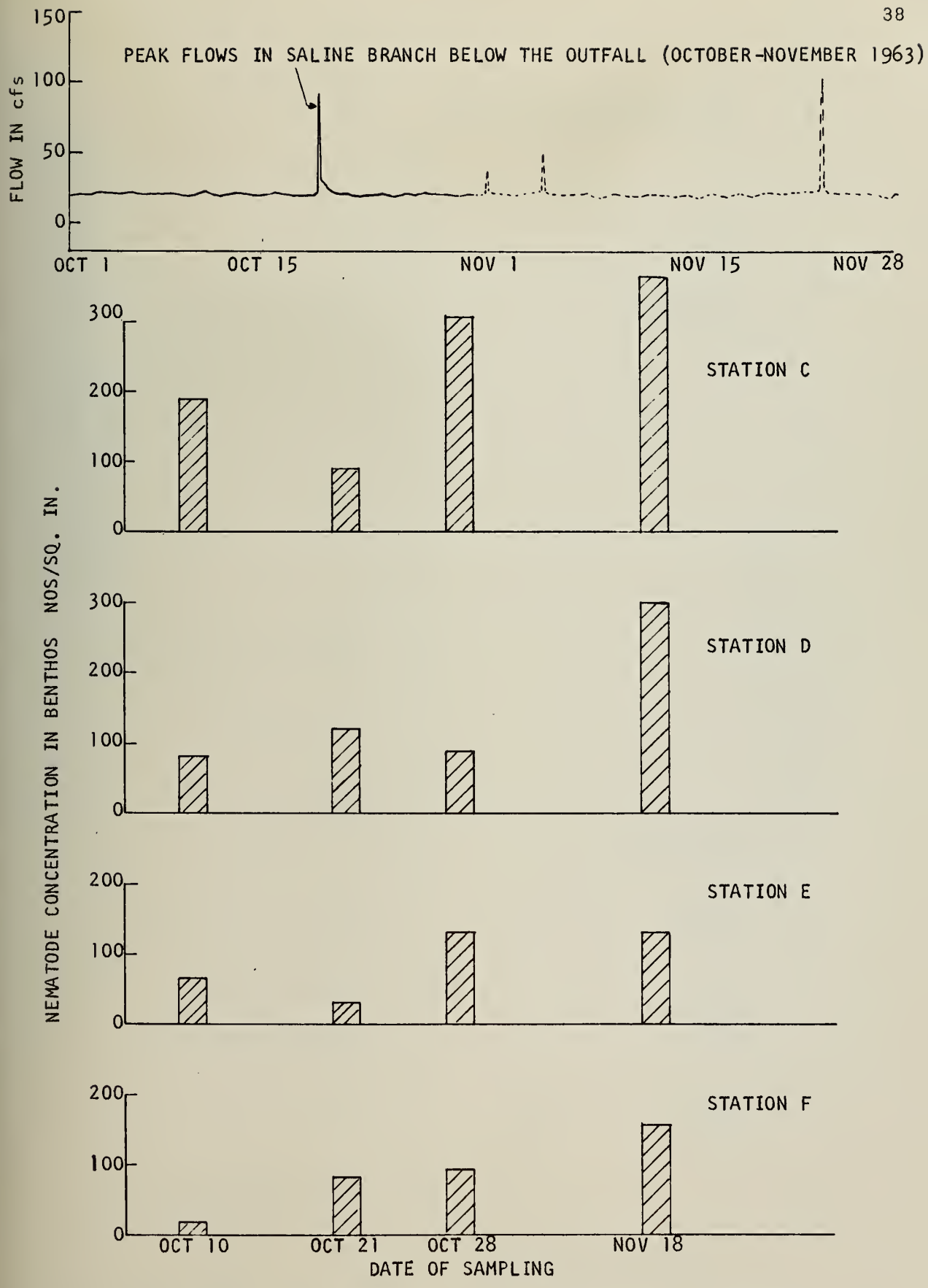


FIGURE 8 VARIATIONS OF NEMATODE CONCENTRATION IN BENTHOS WITH PEAK FLOWS AT EACH STATION -- C THROUGH F (OCTOBER-NOVEMBER 1963)

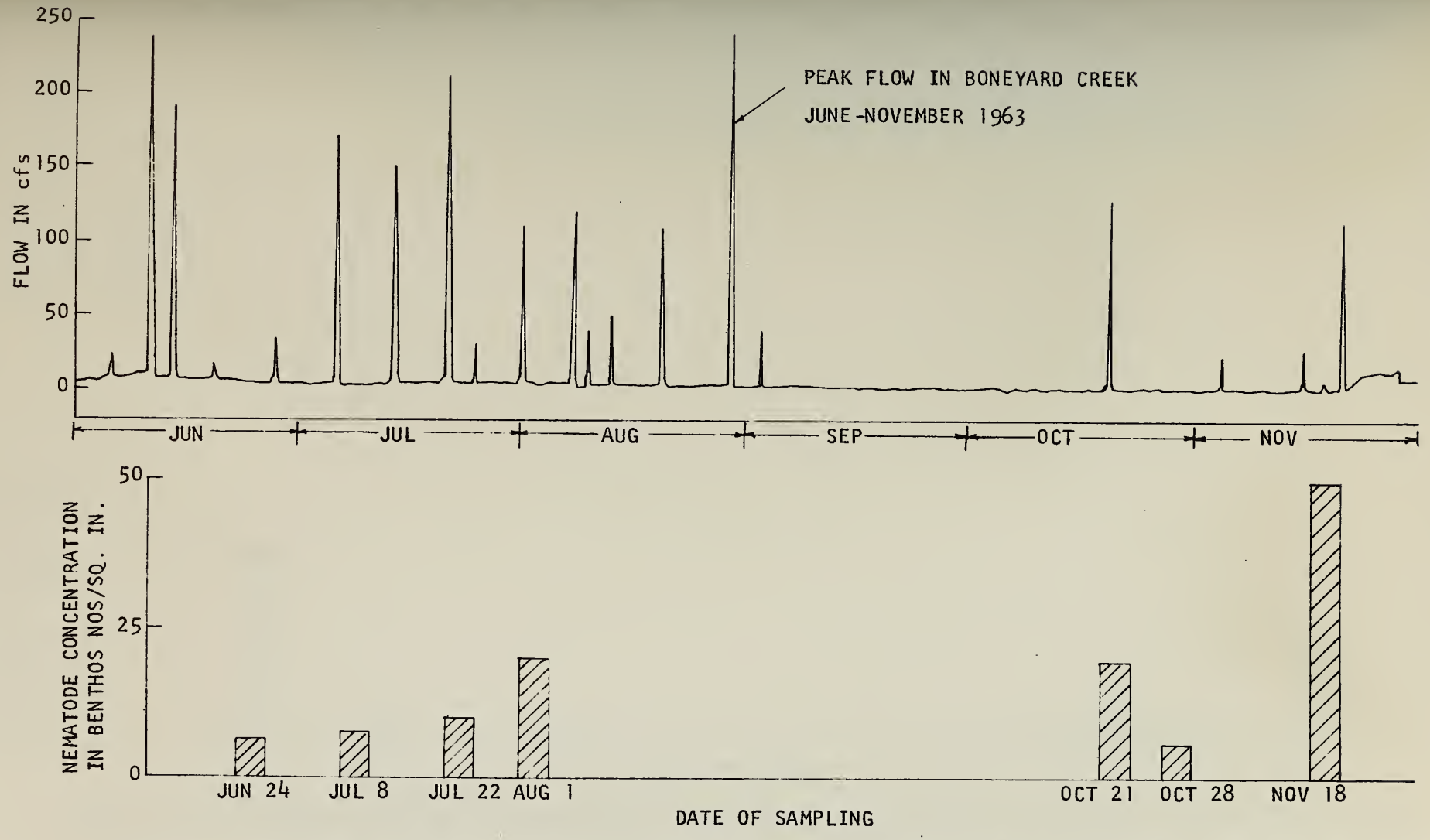


FIGURE 9 VARIATIONS OF NEMATODE CONCENTRATION IN BENTHOS WITH PEAK FLOWS AT STATION A (JUNE-NOVEMBER 1963)

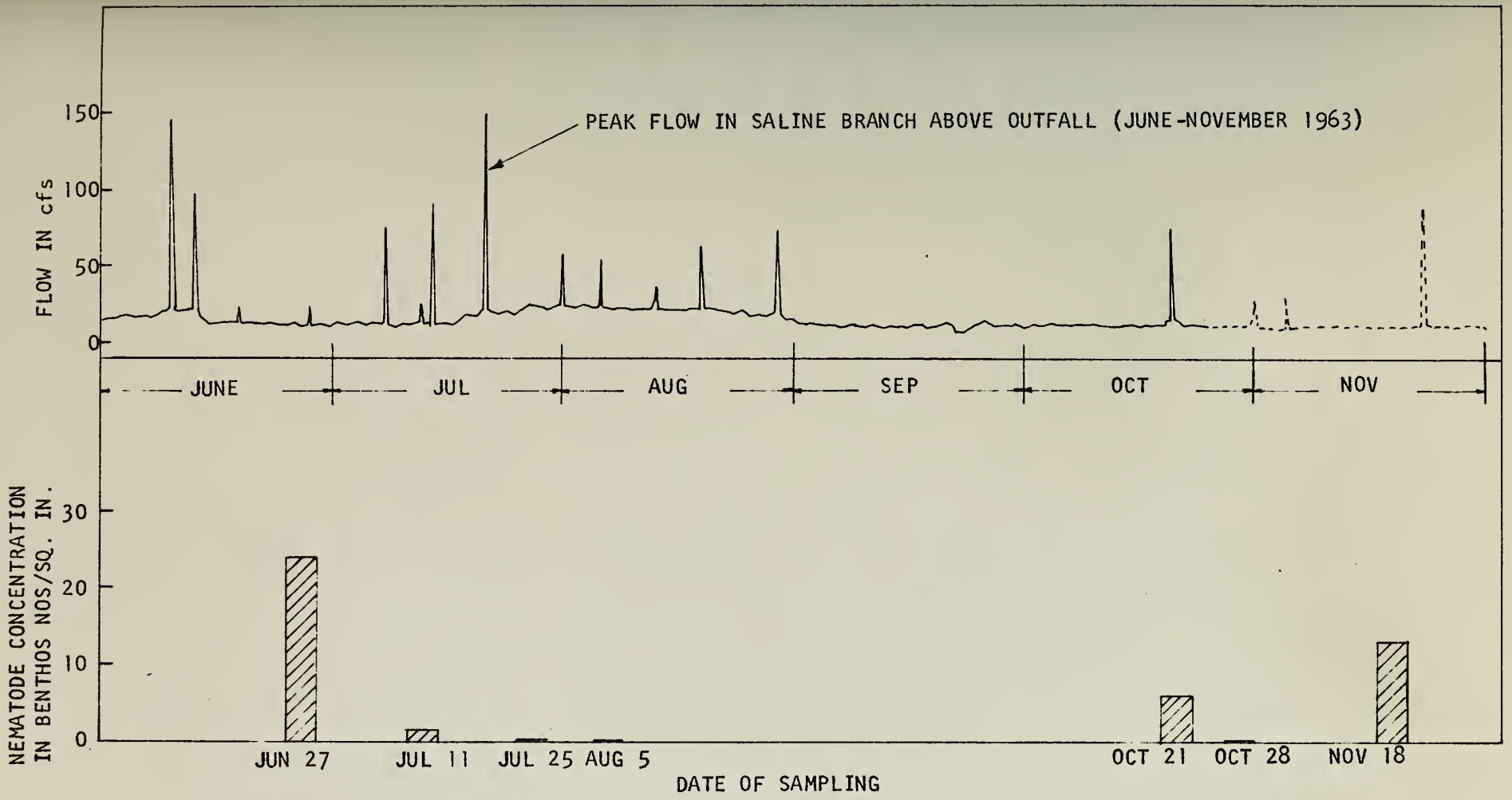


FIGURE 10 VARIATIONS OF NEMATODE CONCENTRATION IN BENTHOS WITH PEAK FLOWS AT STATION B (JUNE-NOVEMBER 1963)

flows on July 13 and 20 practically washed the bed free of nematodes, as shown by the very low concentrations. A series of high flows subsequently prevented any buildup of the nematode concentrations in the benthos. Figure 5 shows the general trend of all Stations C through F, and Figure 6 shows each station separately. The nematode concentrations in Figure 6 are shown as hatched vertical columns, with the dates of sampling marked. Due to the persistent rains, the sampling was discontinued after the middle of August, 1963. However, when sampling was resumed in the fall, October through November, 1963, the significance of the high flows was further illustrated. Figures 7 and 8 show the data for stations C through F, for the period of October-November, 1963. The effect of the high flow of October 19 can be seen in that it reduced the concentrations of nematodes at certain stations similar to the observations made during the summer periods. However, the concentrations increased after this high flow at all stations. The smaller flows of October 30 and November 4 had only a slight effect on the number of nematodes because the flows were not large enough to cause erosion of the beds. Figure 7 shows these features for all the stations together, and Figure 8 shows the variations at each station individually.

Since stations A and B are on a different stretch of the stream system, with different peak flows, separate figures have been prepared to show the conditions at these stations. Figure 9 shows the conditions of flows in the Boneyard Creek, as applicable to Station A. Here the bottom concentrations appear to remain fairly constant even after high flows during the June-August, 1963, period. This may have been due to the fact that, since the Boneyard Creek originates in Champaign, with a relatively small drainage area, the peak flows are relatively short-lived and deposition of the suspended material from the flood water begins settling immediately after the flood.

This silt having been scoured some distance upstream, has the nematodes from that region, and these settle soon after the peak flow has passed. However, the growth of nematodes in the absence of high flows can be seen from observations made during the October-November, 1963, period.

Figure 10 shows the conditions for Station B on the Saline Branch above the outfall of the waste treatment plant. This follows the same pattern of variation as was observed for Stations C through F. The peak flows of the summer period scoured the nematodes from the bed and increase in number of nematodes took place in the absence of peak flows during the October-November, 1963, period. In this respect, the Boneyard Creek and the Saline Branch both above and below the outfall behave differently, chiefly because of the drainage areas being different. In other words, whereas the nematode concentrations in Stations B through F are mostly a result of settling and growth in the benthos, the nematode concentrations at Station A appear to be also due to deposition along with silt in the wake of a high flow.

Another environmental factor considered was pH. Throughout the investigation, the pH measurements showed a range from pH 6.8 to pH 8.0. This range is well within the general range established by Chaudhuri (18), who found a pH range of 5.0 to 9.0 to be suitable for growth of the two predominant species. Dissolved oxygen was never observed to be limiting as far as the stream water was concerned, even though the supersaturated conditions at Station E is thought to be partly responsible for a high concentration during the June-August, 1963, sampling. The average values of effective size and uniformity coefficient as given in Table II indicate no great variation from station to station, for the stream system under investigation. Therefore, these physical properties are not considered as ecological factors of any importance as far as this investigation of nematodes was concerned. However,

it should be recognized that such properties do constitute significant ecological factors when encountered in widely varying values. Chemical oxygen demand and the total nitrogen results on the bottom samples for the period June-August, 1963, are shown in Table II. These chemical tests were made in accordance with Standard Methods (20), except that a known amount of oven-dried sample was taken (one to two grams) instead of a sample in the liquid form, and the results are expressed as milligrams of oxygen or nitrogen per gram of bottom material. Figures 11 and 12 respectively show the variations in nematode counts at stations below and above the waste treatment outfall with respect to the chemical properties of the bottom material. In both of these figures, it is seen that there is no correlation between the nematode concentrations and Chemical Oxygen Demand and total nitrogen. Both of these determinations indirectly indicate the amount of organic pollutants present which might be available for life. It should be taken to mean that the amounts present are above limiting concentrations.

SPACIAL DISTRIBUTION OF NEMATODES

In all the sampling reported above, the samples were obtained from the main course of flow of the stream. A study was undertaken to observe the variations in nematode concentrations in the benthos, both horizontally and vertically at each sampling stations.

Cross-sectional Distribution. Samples were collected with the scoop at five places across a cross-section. Two of these were within one to two feet of either bank and the other three approximately equally spaced in between. The samples collected were analyzed for nematode concentration and pH. The horizontal cross-section was mapped by measuring the water depth at a number of places across the cross-section. The results of the tests

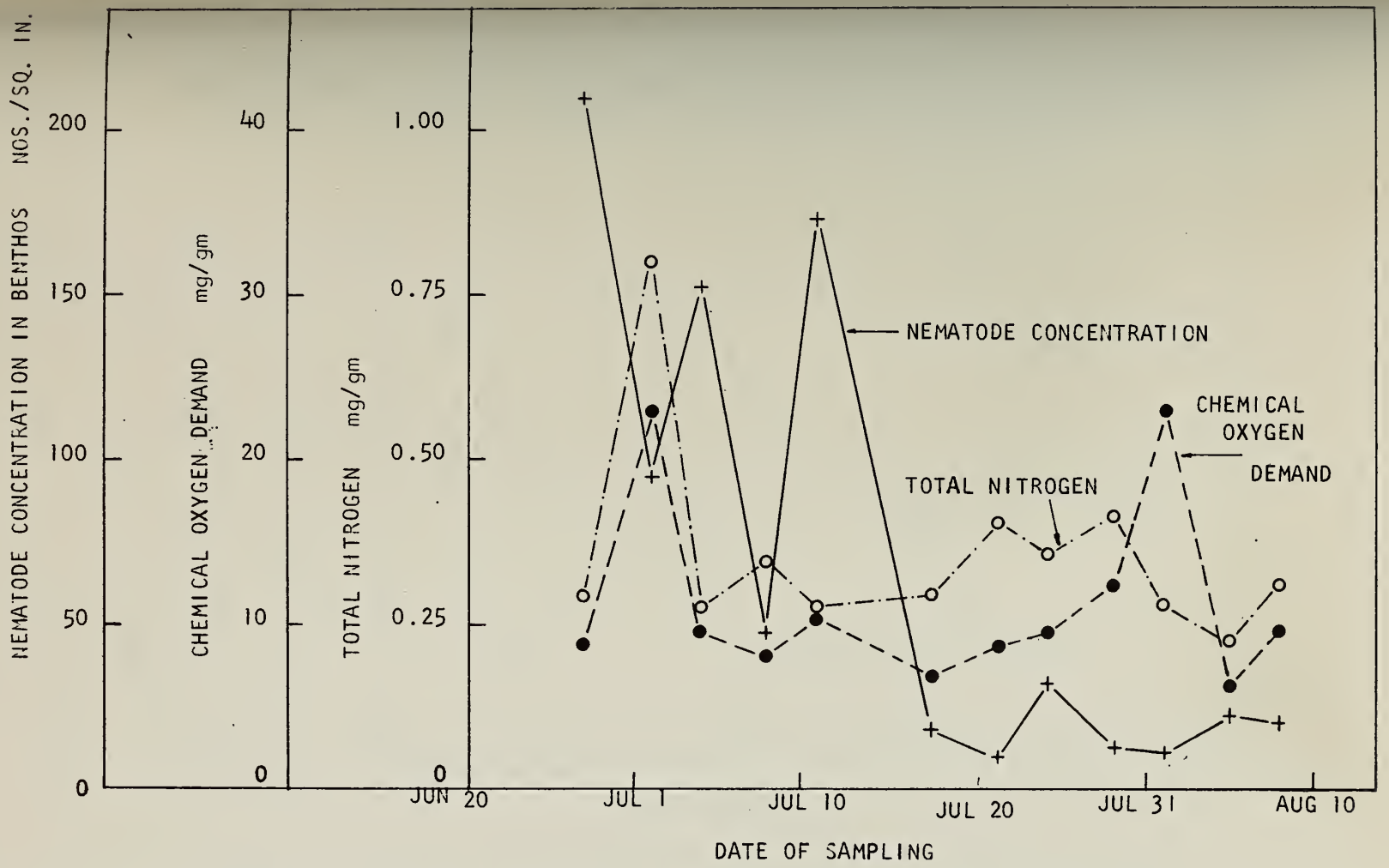


FIGURE 11. NEMATODE CONCENTRATION IN THE BENTHOS vs CHEMICAL PROPERTIES OF THE BOTTOM MATERIAL - STATIONS C THROUGH F (JUNE TO AUGUST 1963)

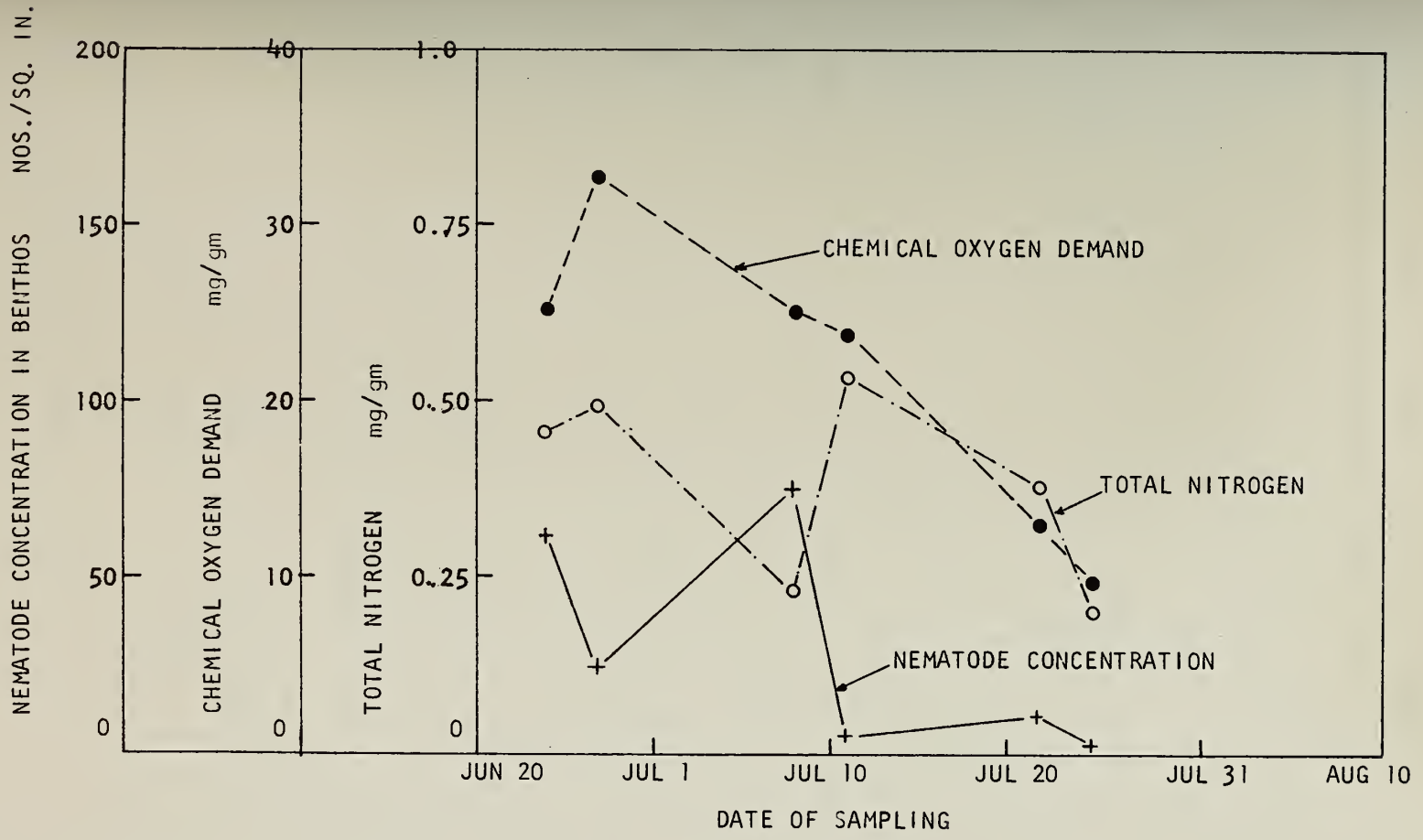


FIGURE 12 NEMATODE CONCENTRATION IN THE BENTHOS vs CHEMICAL PROPERTIES OF THE BOTTOM MATERIAL - STATIONS A AND B (JUNE TO AUGUST 1963)

together with the cross-section for each Station, A through F, are shown in Figures 13 through 18. The dates of sampling of each station are marked on the figures. In general it can be seen from the figures that the nematode concentrations were higher nearer the banks of the stream. An important ecological difference between the littoral and channel regions of the stream is the velocity of flow. Therefore, these observations conform with the earlier conclusion drawn from Figure 3, with regard to Station E, where a high concentration of nematodes was observed at a place having the least velocity of flow. Inconsistent results may be seen in Figure 14 for Station B and Figure 17 for Station E. In the former case, the left bank consisted mainly of rocks and boulders which made sampling very difficult, and hence may have been responsible for low values. In Figure 17, the entire cross-section at Station E was made up of two main stream-lines of flow, with a hump in the center. Accordingly, the stream at this location had a sand-bar running longitudinally in the center where the velocities were sluggish. In other words, there were two streams with the nematode concentrations being higher at either bank and at center and lower concentrations in the regions of the main flows. Figures 13 and 14 show Stations A and B, both of which are located above the outfall. As such, the nematode concentrations in the benthos at these stations in general were low. Therefore the variations of nematode concentration in the benthos from the littoral and channel regions of the stream were not as marked as at other stations. Nevertheless, the same general pattern of cross-sectional distribution can be seen. The higher concentrations nearer the banks or wherever sluggish velocities occur support the view that the absence of currents is conducive for better settling, greater growth and absence of scouring of nematodes in the stream benthos.

STATION A
AUGUST 15, 1963

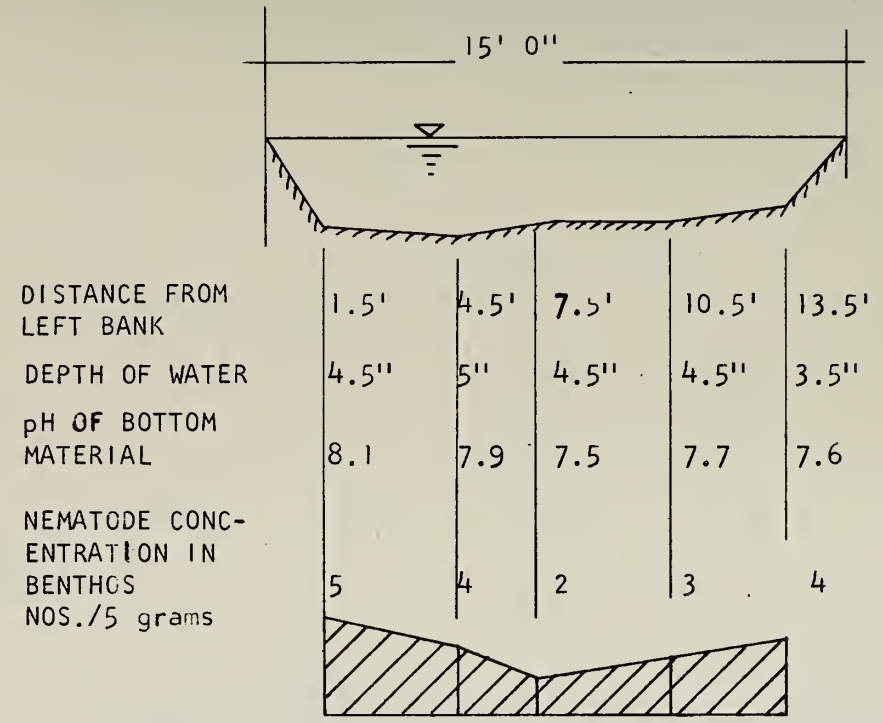


FIGURE 13 CROSS-SECTIONAL DISTRIBUTION OF NEMATODES IN THE BENTHOS AT STATION A

STATION B
SEPTEMBER 12, 1963

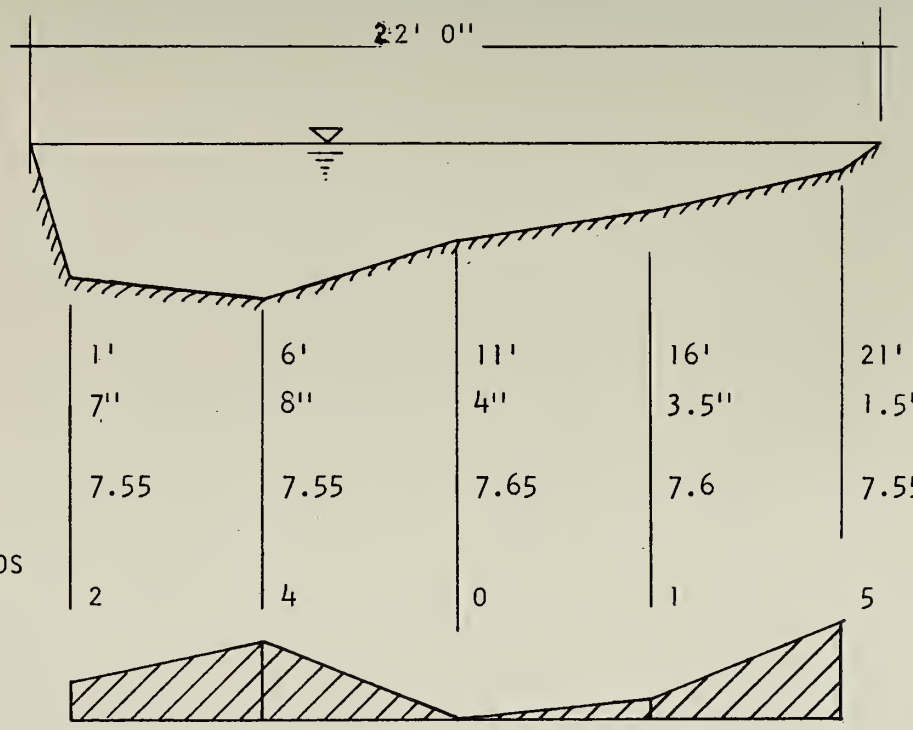


FIGURE 14 CROSS-SECTIONAL DISTRIBUTION OF NEMATODES IN THE BENTHOS AT STATION B

STATION C
AUGUST 15, 1963

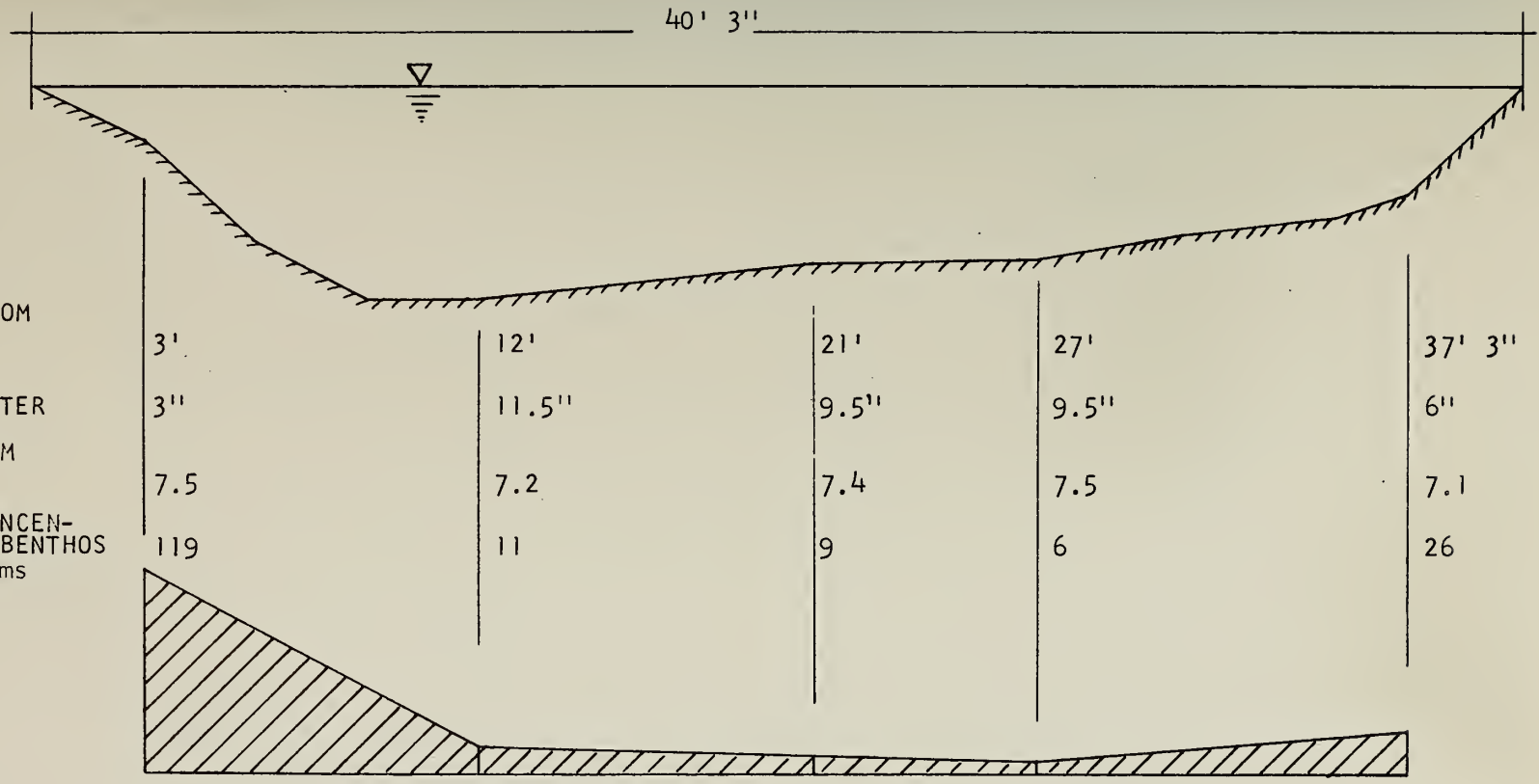


FIGURE 15 CROSS-SECTIONAL DISTRIBUTION OF NEMATODES IN BENTHOS AT STATION C

STATION D
SEPTEMBER 12, 1963

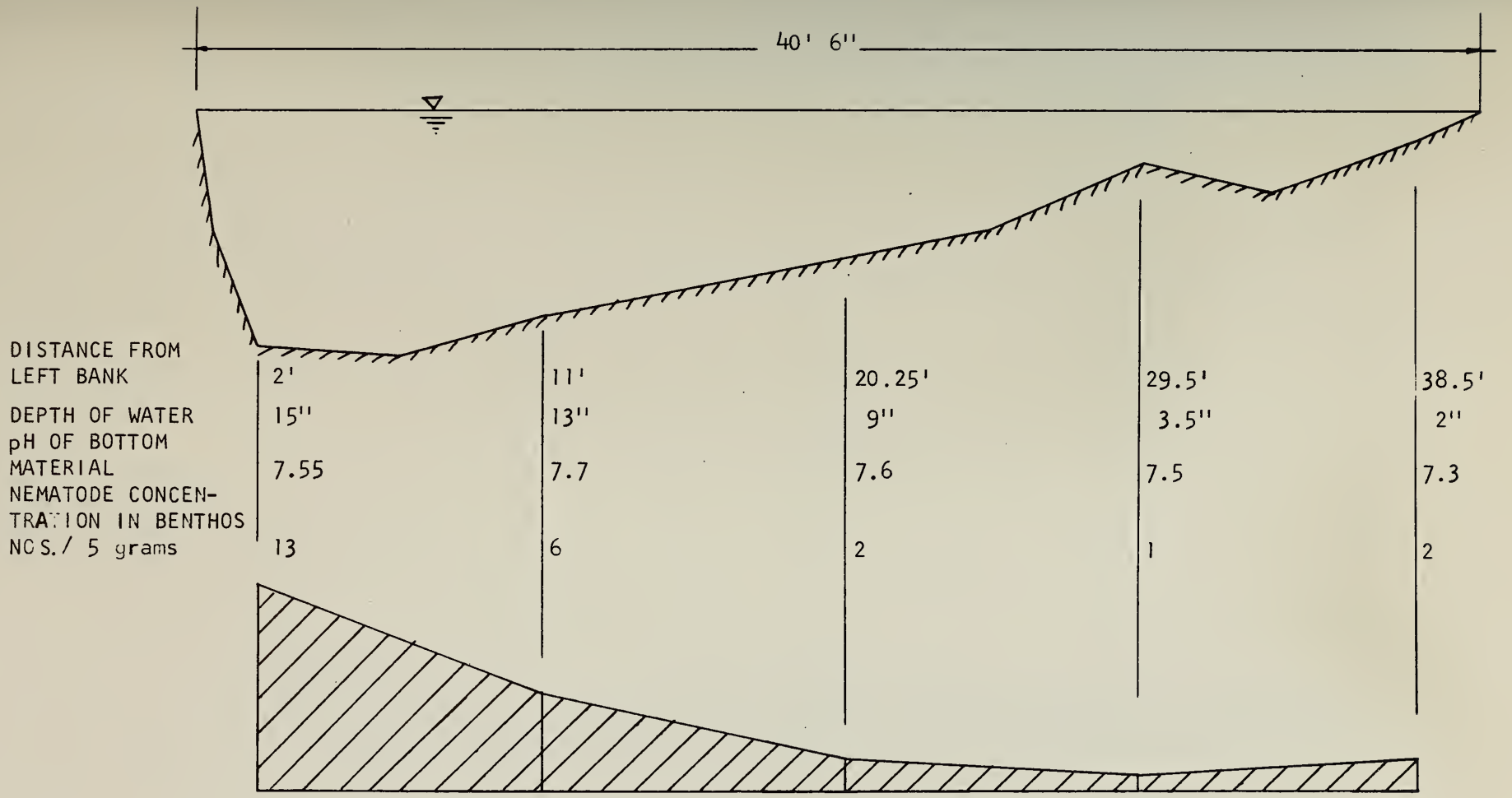


FIGURE 16 CROSS-SECTIONAL DISTRIBUTION OF NEMATODES IN BENTHOS AT STATION D

STATION E
AUGUST 21, 1963

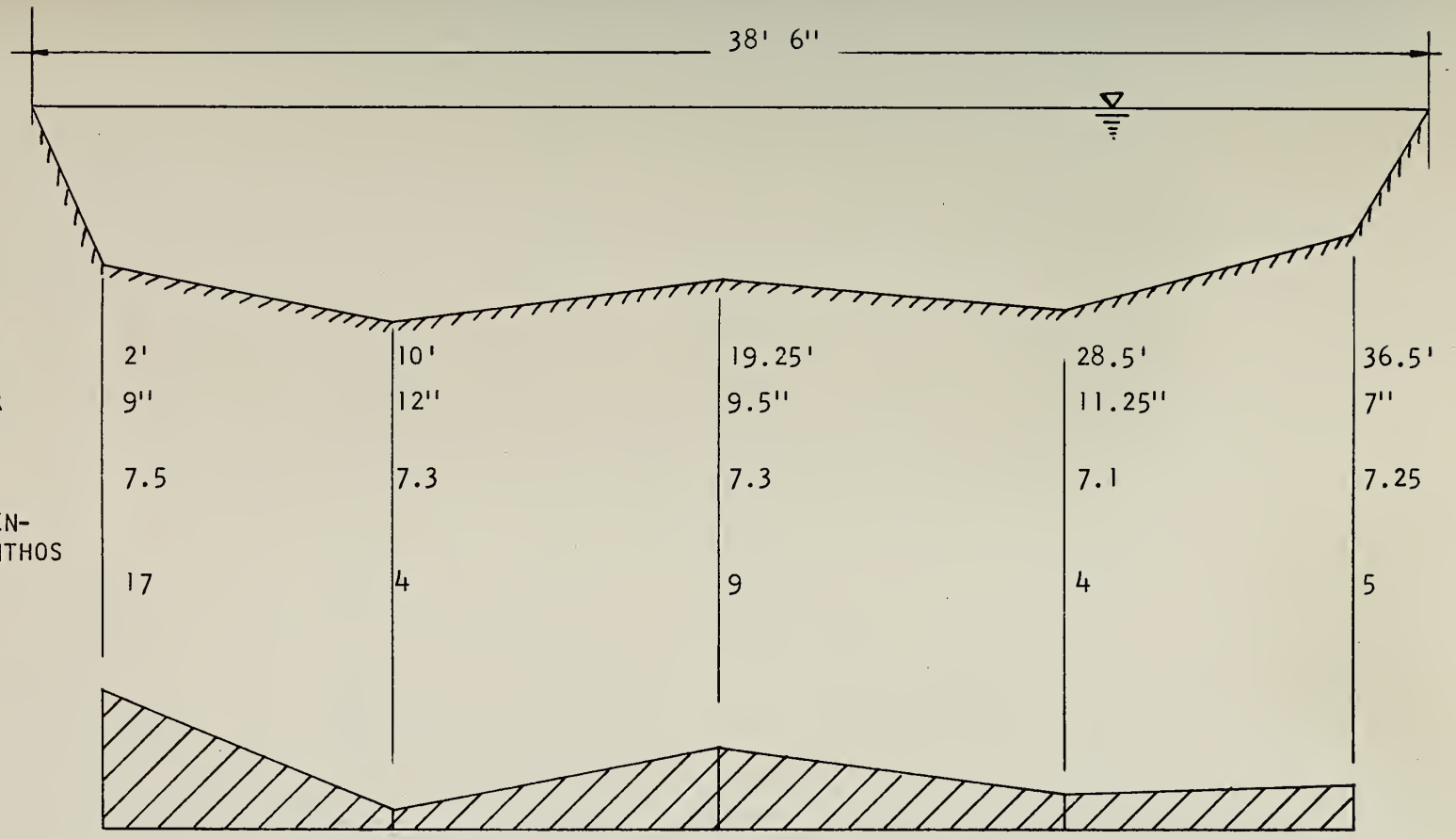


FIGURE 17 CROSS-SECTIONAL DISTRIBU T ION OF NEMATODES IN BENTHOS AT STATION E

STATION F
AUGUST 21, 1963

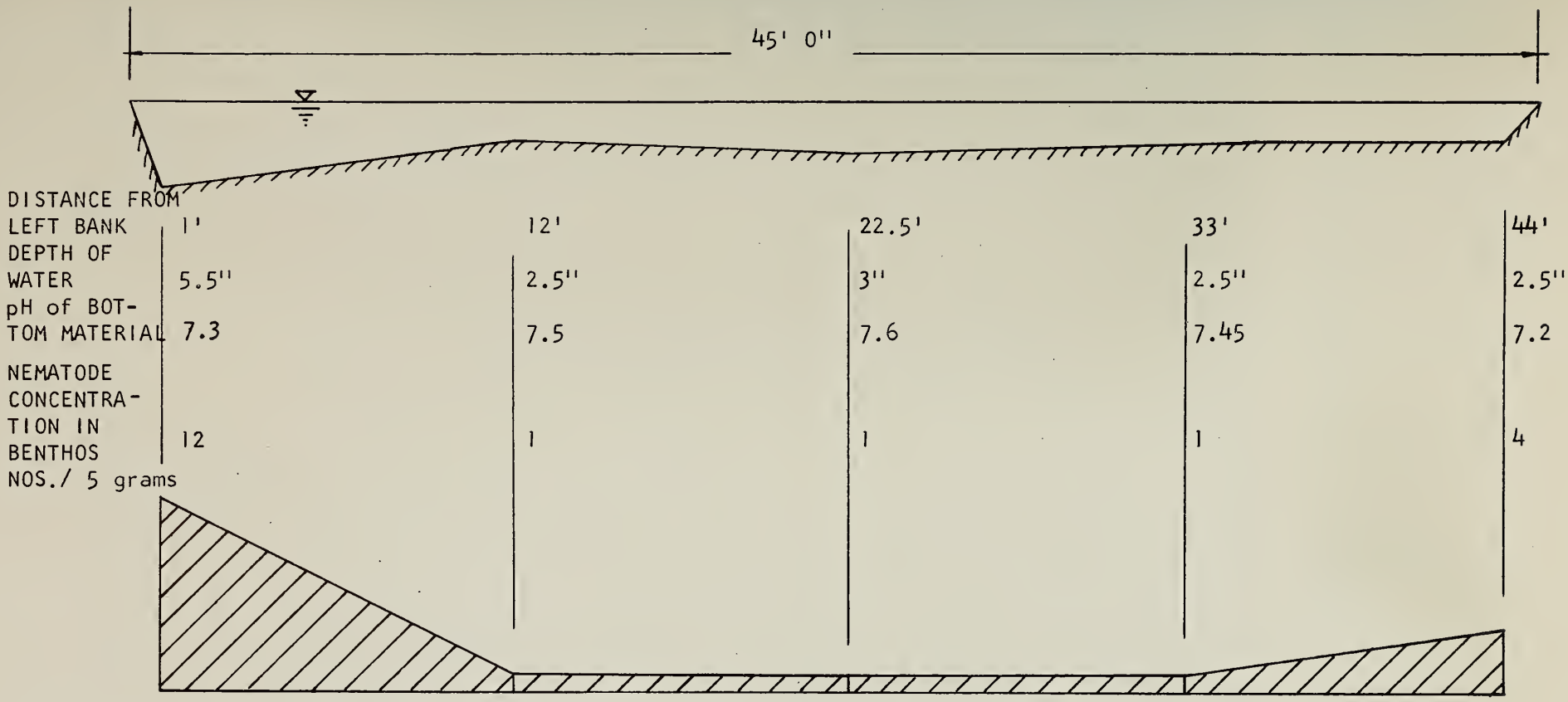


FIGURE 18 CROSS-SECTIONAL DISTRIBUTION OF NEMATODES IN BENTHOS AT STATION F

Vertical Distribution. This aspect of the study had two objectives: (a) to observe the oxidation reduction potential of the benthos at different depths with a view of determining the oxidation-reduction conditions in the benthos, and (b) to observe the distribution of nematodes in a vertical section of the bed.

The oxidation reduction potential measurements were made using platinum and calomel electrodes and a Leeds and Northrup voltmeter. The meter was standardized each time before use with a solution of quinhydrone in pH 4.0 buffer. The electrodes were first placed in the supernatant water in the scoop for the measurement of its potential. Then the electrodes were inserted into the undisturbed sample in the scoop to the desired depth, and readings were taken every three minutes till the reading reached a constant value, which took approximately 30 minutes. The depth of electrodes was then changed as desired and potential measured as before. All these measurements were made in the field immediately after the sample had been collected. The pH of the samples were also measured. The measured values of potential were then corrected for pH to obtain the E_7 values by adding +60 millivolts for every pH unit decreased. The E_7 values for all the samples of each station are shown in Table III. The significance of the E_7 values is that a negative value suggests a completely reduced state of the environment, and a positive value indicates an oxidized state. The greater the magnitude of the positive value, the greater the probability of having free oxygen available. For the samples collected from Stations A through F during June-August, 1963, the E_7 values are shown in Table III and average values are plotted in Figures 19 and 20, which show that for Stations C through F, the oxidation reduction potential remains positive, even at a depth of two centimeters below the mud water interface. This suggests the possibility of the presence of nematodes,

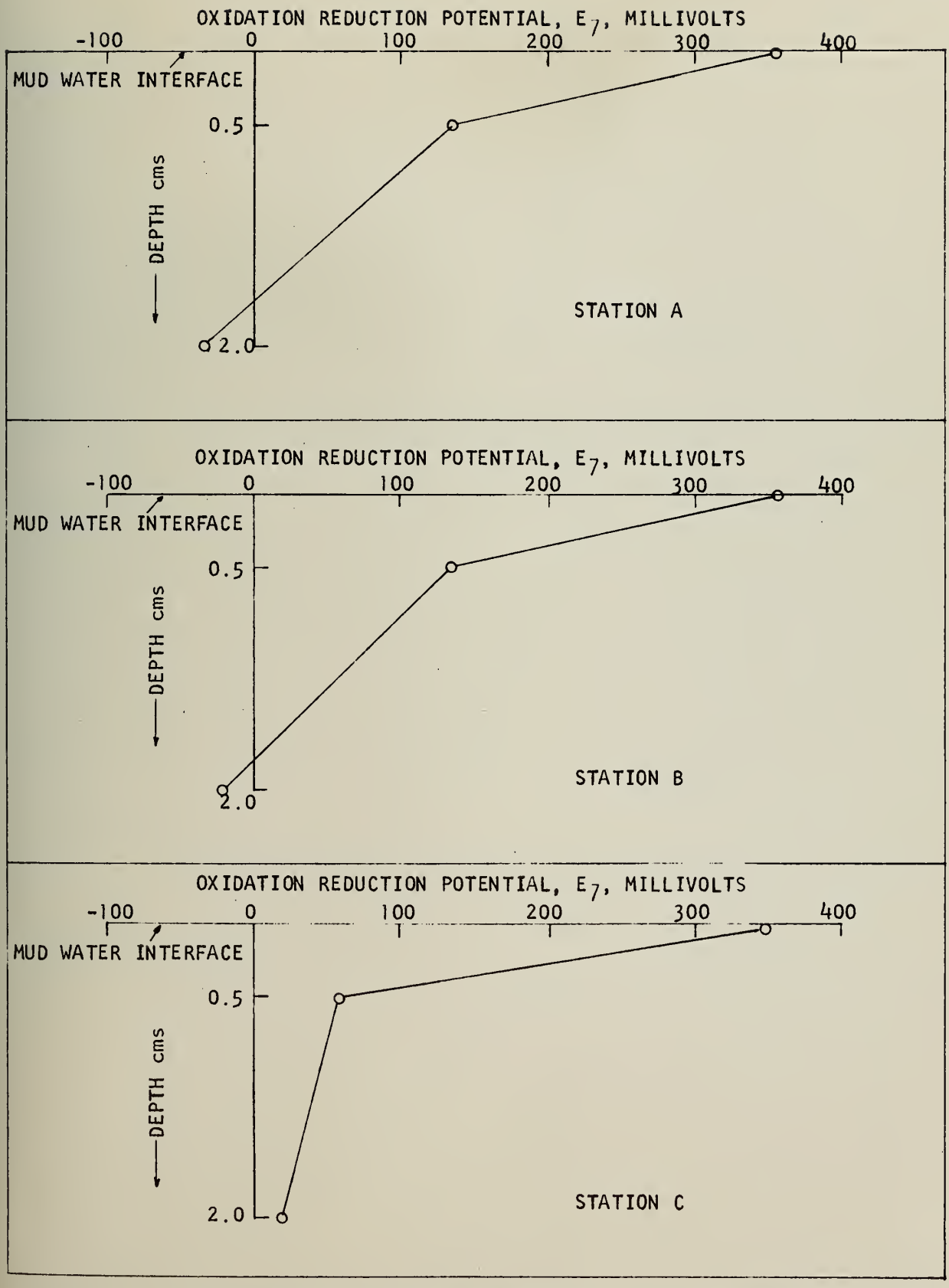


FIGURE 19 AVERAGE OXIDATION REDUCTION POTENTIAL, E_7, IN A VERTICAL SECTION AT STATIONS A THROUGH C (JUNE-AUGUST 1963)

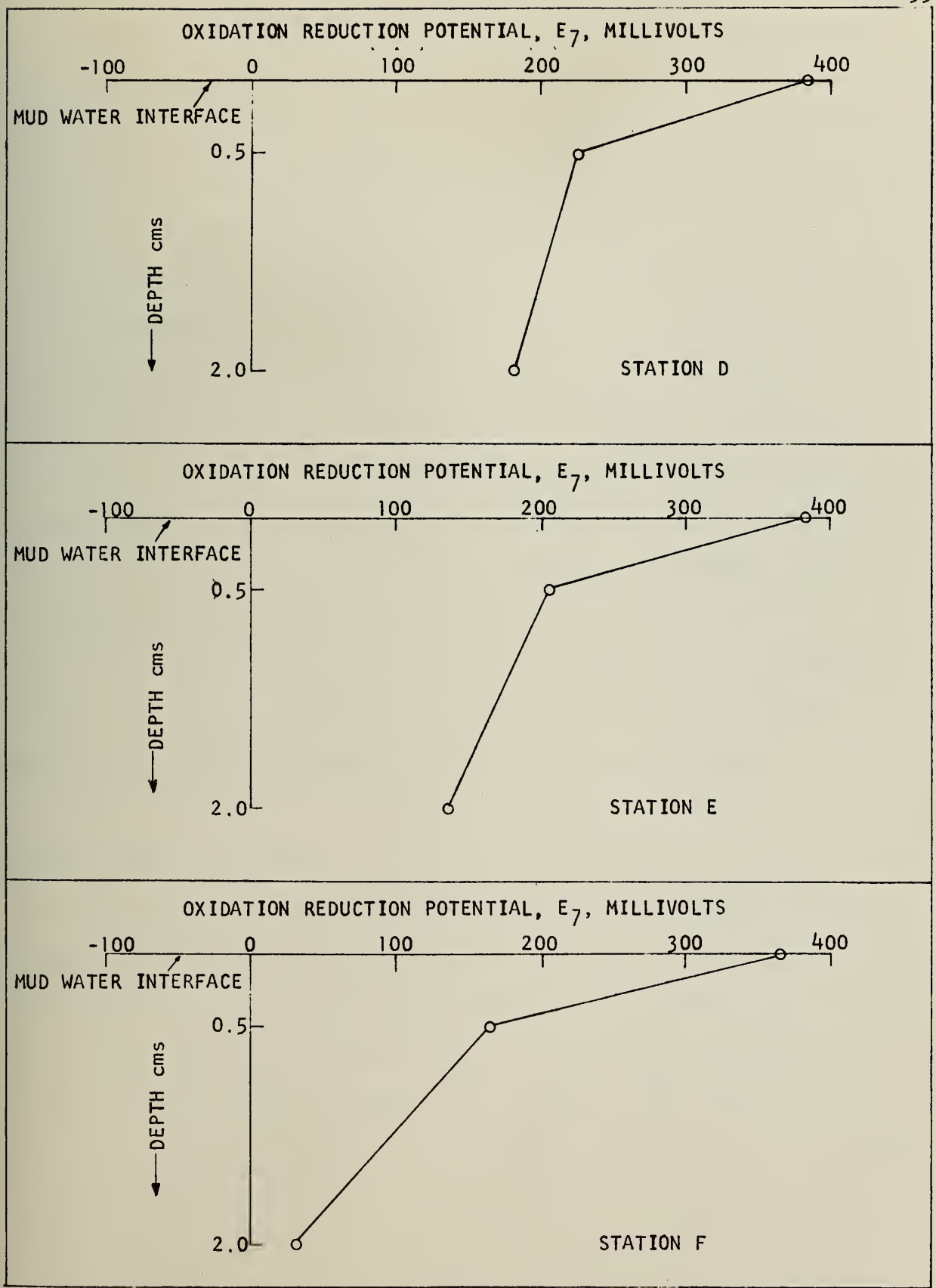


FIGURE 20 AVERAGE OXIDATION REDUCTION POTENTIAL, E_7, IN A VERTICAL SECTION AT STATIONS D THROUGH F (JUNE-AUGUST 1963)

as will be shown to be true later. The bottom material was generally in an oxidized state as shown by the positive values, which meant the absence of complete anaerobic conditions. Therefore, studies were made to observe the relative numbers of nematodes with each centimeter depth of the bottom mud, below the surface. After obtaining an undisturbed sample in the scoop, the sample was drained. The portion within the scoop was then halved, wasting one half and exposing the vertical side of the other half. Aliquots were then obtained from each centimeter depth of this exposed half, and analyzed for nematodes. Results of two such studies are given in Figure 21, relating to Stations E and D. The percentage nematodes in each centimeter depth were then plotted vertically and also the cumulative percentages. It is seen that about 70 percent of the nematodes within the top 4 centimeters of bottom material were concentrated in the top two centimeters, where the conditions had been shown to be more favorable by the oxidation reduction potential measurements. The samples were analyzed for a depth of four centimeters, this being the average depth of samples collected during all the sampling program.

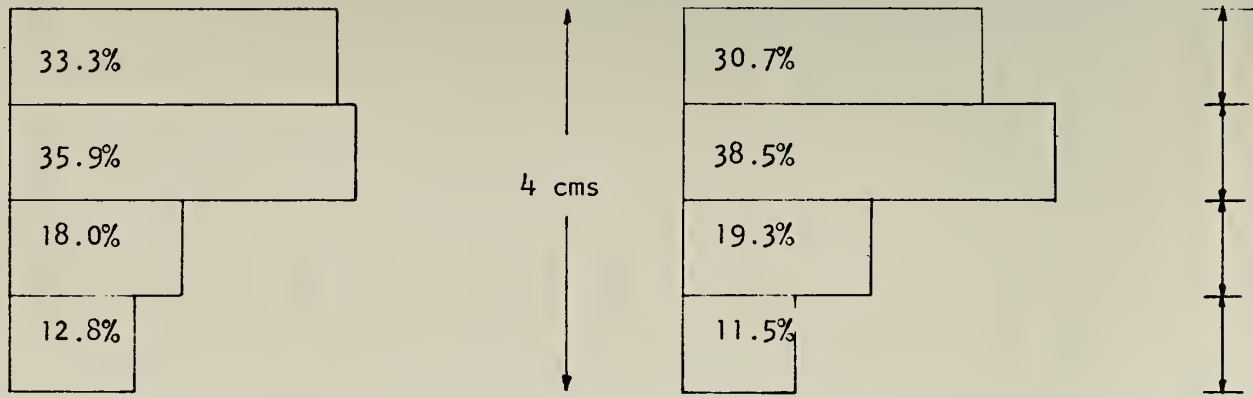
LABORATORY GROWTH OF NEMATODES

The above studies were made to investigate the ecological factors as observed directly in the field. Two factors, namely, temperature and currents, appeared to be the most important factors. In this phase of the study, it was desired to observe the effects of temperature on the nematode concentrations. The samples collected from the field were divided into two portions after mixing them gently and thoroughly. Each portion of the samples was kept in a beaker with sufficient water to submerge the sample. At the time these samples were collected in the field, the temperature variation was between 15° to 20°C. One set of samples was kept at room temperature of

STATION E
NOV. 13, 1963

DEPTH OF SAMPLE	NOS. OF NEMATODES/ 5 gms
1 cm	26
2 cm	28
3 cm	14
4 cm	10

PERCENT NEMATODE IN EACH CENTIMETER DEPTH OF SAMPLE



STATION D
DEC. 9, 1963

DEPTH OF SAMPLE	NOS. OF NEMATODES/ 5 gms
1 cm	8
2 cm	10
3 cm	5
4 cm	3

CUMULATIVE PERCENT

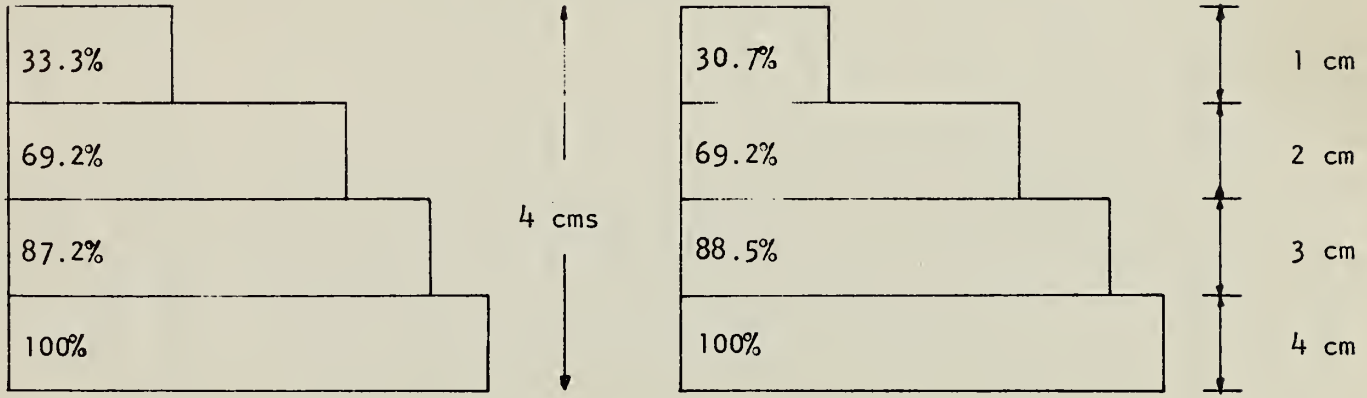


FIGURE 21 VERTICAL DISTRIBUTION OF NEMATODES IN 4 cm DEPTH OF SAMPLES

approximately 25°C, the nematodes being incubated at a higher temperature than in their natural environment. Another set of samples was kept in a cooler maintained at temperature varying from 8° to 10°C, the nematodes in this case being exposed to colder temperatures than their natural habitat. The nematode concentrations in the samples were determined initially and periodically after a number of days of exposure to the particular temperature range. During sampling, the supernatant was carefully decanted, the contents of the beakers mixed gently and thoroughly, and a five-gram aliquot taken for analysis. The supernatant was then returned to the beaker. The beakers were kept under observation for as long as 14 days in the case of the samples incubated at 8° to 10°C in the colder temperature, and 10 days in the case of the samples kept at room temperature. Results for incubation at the higher temperatures are given in Table VI and those in case of colder temperature, are given in Table VII in the Appendix. Under both conditions, there was no increase in number of nematodes; on the contrary, a continuous decrease in nematode concentrations was observed. The percentage survival of nematodes was calculated and the results included in Tables VI and VII of the Appendix. These data are shown in Figure 22. It can be seen from the results that there was greater survival when nematodes were exposed to a colder temperature than when they were exposed to a higher temperature.

An attempt was then made to determine if nematodes, kept under a favorable temperature, 20°C, would grow in a mixed biological culture on an actual sample of bottom material obtained in the field. Growth studies using pure culture have been reported in the literature, but investigations on mixed culture growth have not been reported. The procedure was as follows: two enamel trays of 9" x 15" x 3" size were taken and filled with about a one half inch thick layer of actual stream bed material properly seived through a No. 10

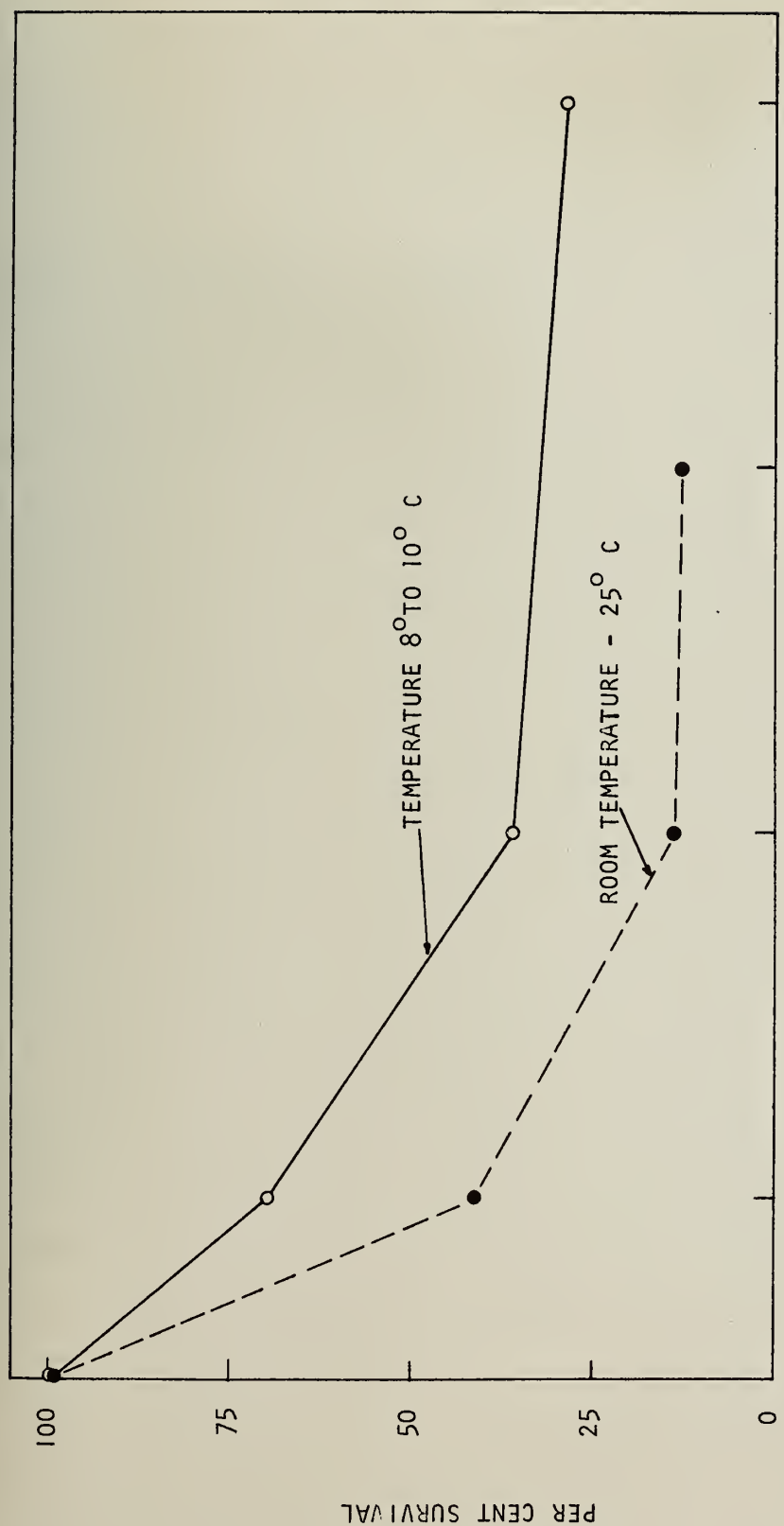


FIGURE 22 PER CENT SURVIVAL OF NEMATODES WITH TEMPERATURE

seive so as to exclude particles larger than two millimeters. These trays were kept at a temperature of about 10°C, with the bottom material submerged under stream water for about two weeks. At the end of this period, the nematode concentration in the trays had fallen to a very low value. The water from the trays was completely drained. Two pure cultures of nematodes, one each of Diplogaster nudicapitatus and Diplogasteroid sp. were used to inoculate each of the two trays. For tray No. 1, growing cultures of the two nematode species were mixed and diluted in a liter of water in a beaker which was filtered through a membrane filter. The membrane filter was rinsed thoroughly to wash the nematodes free of the culture fluid and antibiotics. The worms recovered from the filter were then resuspended in about 100 ml of water. This suspension was then mixed with the contents of the tray No. 1, taking care to mix it gently, but thoroughly. For tray No. 2, two growing culture flasks including the culture fluid were directly suspended in about 100 ml of water and the suspension was mixed gently but thoroughly with the contents of the tray. It should be mentioned that the culture fluid contained a considerable amount of antibiotics, which were added to tray No. 2 but not to tray No. 1. Both trays were maintained at a temperature of 20°C, maintaining a fairly constant moisture content in the bottom material. The nematode concentrations initially and subsequently, at an interval of about every two days, were determined by taking aliquots from the trays. Each day's determination was an average of four random samples from each tray, so that local variations within the tray could be averaged to represent the condition in the whole tray. For each tray, the results obtained are shown in Table VIII of the Appendix. The concentration on any given day of analysis is expressed as a percentage of the initial day concentration, and these percentages are also shown in the table. A figure showing these data is given in Figure 23.

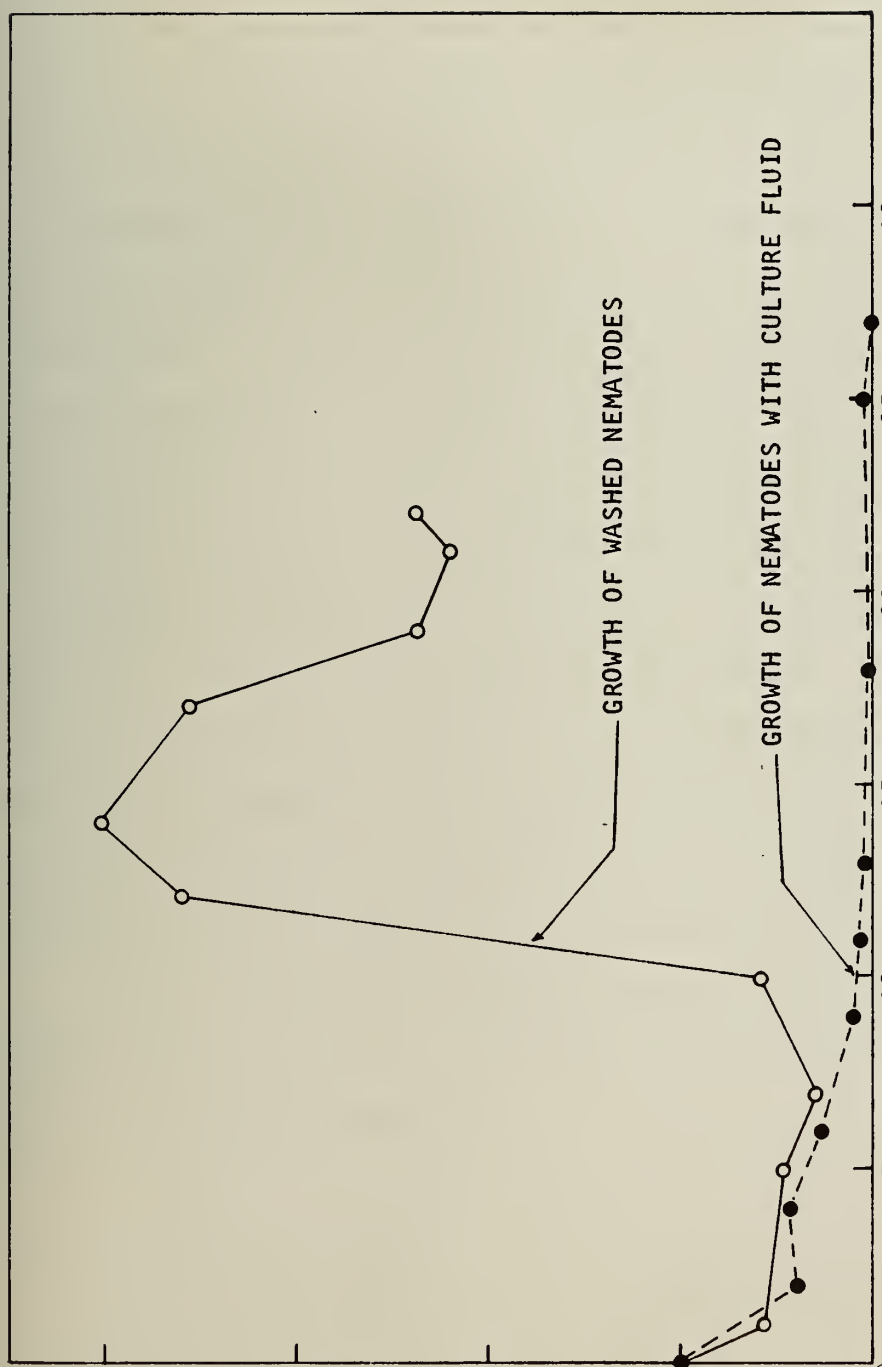


FIGURE 23 GROWTH CHARACTERISTICS OF NEMATODES IN A MIXED BIOLOGICAL CULTURE IN A HETEROGENEOUS MEDIA, AT 20°C

Tray No. 1, containing the washed nematodes, had a lag period of about seven days after which a typical growth curve for microorganisms was observed. This lag period may have been due to the fact that the nematodes from the culture flasks took time to acclimate to the heterogeneous substrate in the tray after having been removed from artificial media of the culture flasks. The maximum concentration was observed to be on the 14th day, after which the numbers decreased. This decrease may have been due to the disappearance of the nematodes born at the beginning of the log phase or due to oxygen becoming limiting. An entirely different result was observed in tray No. 2. The nematode concentration continuously decreased. This fact was attributed to the antibiotics in the culture fluid that had been mixed with the substrate in this tray. The antibiotics had apparently prevented the growth of bacteria in the substrate, thus starving the nematodes. The cultural fluid itself, though devoid of bacteria, is a good food for nematodes. However, when it was diluted and mixed with the substrate in the tray, it was not immediately available to the nematodes. Since nematodes cannot feed on particulate matter, in the absence of bacteria, they did not survive. This observation illustrates that the food habits of nematodes are related to bacteria as a source of food.

Growth of nematodes in mixed biological culture in a heterogeneous media was possible in tray No. 1, but was not possible because of a lack of an adequate food supply in tray No. 2.

IV. CONCLUSIONS

The following conclusions were made in light of the results and discussions presented in this study:

1. The modified centrifugal flotation technique using a sugar solution of specific gravity 1.10 for isolation of nematodes from benthic samples has been found to be satisfactory.

2. The effluent from a waste treatment plant has been found to be the principal source of nematodes in the stream benthos investigated, as demonstrated by the fact that the concentrations were significantly higher below the outfall than those above. However, the concentration in the benthos does not follow in proportion to their concentration in water.

3. The concentration of nematodes in the benthos was generally observed to decrease with distance downstream from the outfall.

4. The concentration of nematodes in the benthos decreased considerably following a high discharge, indicating the scouring of nematodes from the bottom material by high flows. The concentrations, however, began to increase in the benthos during low flows.

5. From oxidation reduction potential measurements of the benthic samples, the top two centimeter depth was found not to have anaerobic conditions.

6. The vertical distribution of nematodes in the stream benthos showed a concentration to the extent of 70 percent in the top two centimeters of the benthos. This conformed with the observations made on oxidation reduction potential with the conclusion above.

7. The concentration of nematodes across the width of the stream was observed to be least in the region of the main flow of the stream and

was highest near the banks, supporting the view that absence of currents contributes towards higher concentrations.

8. No correlation between the concentration of nematodes and the physical and chemical properties of the bottom material and the environment was evident from the environmental variations encountered in this study.

9. The concentration of nematodes in the benthos was observed to be higher during cooler months than during the warmer months.

10. Nematodes in the benthic samples from the stream were unable to survive under laboratory conditions when exposed to either substantially higher or lower temperatures than those of their natural environment. However, their survival at lower temperature was greater than that at higher temperature.

11. Growth of nematodes in the bottom material from the stream was found to be possible in the laboratory under a favorable temperature of 20°C. However, the presence of antibiotics seriously prevented their growth probably due to the absence of bacterial food needed by nematodes.

12. From the entire study, currents and temperature were found to be the most important ecological factors affecting the occurrence and abundance of nematodes in the stream benthos.

V. SUGGESTIONS FOR FUTURE WORK

As a result of the studies reported here, the following suggestions for future work are made:

1. The investigation should be extended to sections of the stream farther downstream.

2. A more intensive study over a smaller selected section of the stream would be useful in order to establish the balance of nematodes in water and benthos to illustrate the exchange of these organisms between the two.

3. A study by tracer technique to determine the fate of nematodes discharged in the effluent of waste treatment plants into the stream system.

4. Extensive laboratory studies on the growth characteristics of mixed biological cultures in a heterogeneous media should be made under known environmental conditions of temperature, oxidation reduction potential, aerobiosis, anaerobiosis, etc.

5. Studies on the quantitative aspects of the effect of currents on nematodes in benthos should be continued.

6. A complete survey of all forms of life in the environment of nematodes should be made to establish the biological dependency and relationships.

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VII. APPENDIX

TABLE I

ENVIRONMENTAL CONDITIONS AND NEMATODE CONCENTRATION
IN THE BENTHOS AT STATIONS A-F DURING JUNE-AUGUST, 1963

Sampling Station & Dates	pH	Temperature °C	Dissolved Oxygen mg/ℓ	Dissolved Oxygen Avg.	Nemt./sq. inch No.	Nemt./sq. inch Avg.	Average Velocity fps
A/6-24	7.60	24.3			6.3		
A/7-8	7.80	21.5	5.0		7.5	10.8	0.55
A/7-22	7.70	27.0	3.6	3.7	10.2		
A/8-1	7.70	25.0	2.5		19.2		
B/6-27	7.45	25.8			24.8		
B/7-11	7.60	22.0	7.7		1.6	6.6	1.4
B/7-25	7.67	26.5	11.9	10.4	0		
B/8-5	8.00	25.3	11.7		0		
C/7-4	7.40	22.0	2.8		204.0		
C/7-18	7.53	26.5	5.8	3.7	23.6	63.5	1.4
C/7-29	7.10	27.0	4.2		6.5		
C/8-8	7.30	26.5	2.0		20.0		
D/7-4	7.65	23.5	4.2		102.5		
D/7-8	7.40	24.0	5.1	3.2	48.2	43.1	.60
D/7-22	7.25	24.5	2.4		9.8		
D/8-1	7.60	24.0	1.0		11.7		
E/6-27	7.25	28.8	11.1		210.0		
E/7-11	7.43	23.0	10.6	9.1	173.0	109.4	.25
E/7-25	7.50	25.3	10.8		32.0		
E/8-5	7.40	24.0	3.9		22.6		
F/7-1	7.50	30.0			95.5		
F/7-18	7.40	25.8	5.5		12.9	34.2	1.18
F/7-29	7.45	25.8	8.5	6.4	12.7		
F/8-8	7.40	24.5	5.1		15.6		

TABLE II

PHYSICAL AND CHEMICAL PROPERTIES OF BENTHIC SAMPLES (JUNE-AUGUST, 1963)

Sampling Station & Date	C. O. D. mg/gm of dry sample	Total N mg/gm of dry sample	Effective Size μ microns	avg.	Uniformity Coefficient μc μc	avg.	Nematode Concentration in Benthos nos/sq in
A/6-24	25.40	0.460	300		2.6		6.3
A/7-8	25.55	0.241	335	315	5.45	3.3	7.5
A/7-22	12.88	0.152	335		2.14		10.2
A/8-1	22.35	0.130	290		3.0		19.2
B/6-27	32.95	0.486	260		3.92		24.8
B/7-11	23.60	0.536	450	384	2.54	2.7	1.6
B/7-25	9.96	0.206	385		1.83		0
B/8-5	29.7	0.365	440		2.5		0
C/7-4	11.48	0.350	450		2.54		204.0
C/7-18	8.14	0.323	420	418	1.57	2.0	23.6
C/7-29	6.94	0.285	375		1.76		6.5
C/8-8	11.0	0.326	430		2.21		20.0
D/7-4	8.99	0.195	350		3.14		102.5
D/7-8	8.01	0.344	470	381	3.25	4.2	48.2
D/7-22	8.18	0.410	380		5.26		9.8
D/8-1	23.2	0.262	325		5.23		11.7
E/6-27	8.79	0.281	335		6.12		210.0
E/7-11	10.05	0.264	330	336	2.66	3.4	173.0
E/7-25	9.59	0.369	290		3.1		32.0
E/8-5	6.4	0.221	390		1.54		22.6
F/7-1	22.90	0.810	265		11.1		95.5
F/7-18	5.68	0.265	310	286	1.51	7.3	12.9
F/7-29	17.45	0.46	260		8.10		12.7
F/8-8	8.33	0.316	310		8.55		15.6

TABLE III

OXIDATION REDUCTION POTENTIAL OF SAMPLES (JUNE-AUGUST, 1963)

Station	Date	Oxidation Reduction Potential in Millivolts					
		Supernatant Water		At 0.5 cm depth in Benthos		At 2.0 cm depth in Benthos	
		millivolts	avg.	millivolts	avg.	millivolts	avg.
A	6/24	+142		+52		-64	
	7/8	+80		+7		-127	
	7/22	+709		+267		+142	
	8/1	+484	+353	+208	+133	-87	-34
B	6/27	+234		+71		-53	
	7/11	+92		+28		-48	
	7/25	+550		+328		+141	
	8/5	+539	+353	+101	+132	-127	-22
C	7/4	+153		+14		-51	
	7/18	+295		+173		+168	
	7/29	+501		+217		+88	
	8/8	+445	+348	-167	+59	-117	+22
D	7/4	+289		+219		+199	
	7/8	+292		+215		+195	
	7/22	+485		+358		+266	
	8/1	+476	+385	+111	+225	+63	+181
E	6/27	+237		+184		+141	
	7/11	+316		+250		+246	
	7/25	+497		+142		+72	
	8/1	+484	+383	+251	+206	+97	+139
F	7/1	+318		+56		+59	
	7/18	+148		-74		-109	
	7/29	+506		+243		+133	
	8/8	+488	+365	+268	+163	+38	+31

TABLE IV

NEMATODE CONCENTRATIONS IN THE STREAM WATER

Date	Nematode Concentration at Station -- nos/gallon					
	A	B	C	D	E	F
6/17	68					
6/20			228			148
6/27			203		30	
7/1	45	105				
7/4			325	143		
7/8	45	7		7		
7/11					37	37
7/18			1074		30	22
7/22	15	7		180		
7/25					83	
7/29		15	38			7
8/1				15		
8/5	295					
8/8			15			
8/12	53	15				
8/15			522	408	68	30
Avg.	87	30	343	150	50	49
10/11						
10/14	98	181				
10/18			1453	545	174	8
10/21			6601	5707	302	38
10/25	8					
10/28			4072	4284	454	
11/1	16	0	408	673	423	280
11/6			3480	4118	2308	1203
11/8						
11/9	38					
11/13			8070	5253	2570	1763
11/16	8	23	5238	2944		1196
11/18			7800		2420	1847
Avg.	34	68	4640	3360	1236	905

TABLE V

ENVIRONMENTAL CONDITIONS AND NEMATODE CONCENTRATION
IN THE BENTHOS AT STATIONS A-F DURING OCTOBER-NOVEMBER, 1963

Sampling Station & Date	pH	Temperature °C	Dissolved Oxygen mg/ℓ	Dissolved Oxygen Average	Nemt./sq. inch No.	Nemt./sq. inch Avg.
A/10-21	6.9	23	.6		19	
A/10-28	6.8	17	2.7	1.9	6	25
A/11-18	7.2	15	2.4		50	
B/10-21	7.1	21	3.6		6	
B/10-28	7.2	15	6.2	3.9	0	6.3
B/11-18	7.0	13	1.9		13	
C/10-10	---	21	.8		189	
C/10-21	7.0	24	1.2	1.4	88	236
C/10-28	6.9	20	2.2		302	
C/11-18	7.2	17	1.5		365	
D/10-10		18.5	.9		82	
D/10-21	7.0	24	3.0	2.8	120	146
D/10-28	7.1	19	5.1		88	
D/11-18	7.0	17	2.1		296	
E/10-10	---	17	2.3		63	
E/10-21	7.3	23	3.3	5.8	32	90
E/10-28	7.3	17	10.4		132	
E/11-18	7.4	16	7.0		132	
F/10-10	---	17	2.9		19	
F/10-21	7.4	22.5	3.9	6.5	82	88
F/10-28	7.4	16	10.7		95	
F/11-18	7.3	15	8.7		158	

TABLE VI

SURVIVAL OF NEMATODES AT ROOM TEMPERATURE (25°C)

Sample No.	0-Day Concentration		3-Day Concentration		6-Day Concentration		10-Day Concentration	
	nos/5 grams	Percentage	nos/5 grams	Percentage of 0-day	nos/5 grams	Percentage of 0-day	nos/5 grams	Percentage of 0-day
1	48	100	18	37.5			0	0
2	30	100			4	13.3		
3	14	100	7	50			2	14.3
4	13	100			1	7.7		
5	6	100	2	33.3				
6	10	100			2	20		
7	15	100	7	46.7			4	26.7
Average		100		42		13.7		13.7

TABLE VII

SURVIVAL OF NEMATODES AT COOLER TEMPERATURES (8° to 10°C)

Sample No.	0-Day Concentration		3-Day Concentration		6-Day Concentration		14-Day Concentration	
	nos/5 grams	Percentage	nos/5 grams	Percentage of 0-day	nos/5 grams	Percentage of 0-day	nos/5 grams	Percentage of 0-day
1	58	100					11	18.9
2	16	100	8	50	2	12.5		
3	21	100					6	28.6
4	25	100					18	72
5	10	100	8	80	6	60		
6	47	100					6	12.8
7	5	100	4	80				
8	8	100					1	12.5
Average		100		70		36		29

TABLE VIII

GROWTH CHARACTERISTICS OF NEMATODES IN A MIXED BIOLOGICAL CULTURE IN A HETEROGENEOUS MEDIA AT 20°C

Time days	Tray No. 1 with Washed Nematodes						Tray No. 2 with Nematodes & Culture Fluid					
	Nematodes Concentration/5 gms in Sample					Percent of 0-day Average	Nematodes Concentration/5 gms in Sample					Percent of 0-day Average
	1	2	3	4	Avg.		1	2	3	4	Avg.	
0	10	13	9	17	12.25	100.	48	29	62	33	43.0	100.
1	7	11	4	6	7.0	57.1						
2							19	7	23	19	17.0	39.5
4							6	16	38	17	19.25	44.7
5	5	6	7	4	5.5	44.8						
6							12	5	10	23	12.5	29.
7	7	4	2	2	3.75	30.6						
9							3	6	5	2	4.0	9.3
10	14	5	4	5	7.0	57.1						
11							6	0	1	6	3.25	7.5
12	17	49	62	45	43.25	353.0						
13							1	0	1	1	0.75	1.74
14	33	50	72	42	49.25	402.						
17	60	40	21	53	43.5	355.						
18							1	2	1	2	1.5	3.5
19	26	17	46	27	29.0	236						
21	23	13	32	40	27.0	220.						
22	7	37	9	63	29.0	236.						
25							1	3	3	4	2.75	6.4
27							0	1	0	0	0.25	0.6