PHYSIO-MORPHOLOGICAL EFFECTS OF ABRUPT THERMAL STRESS ON DIATOMS

by

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INTRODUCTION

Increased temperature as a stress factor in aquatic environments has recently received much attention. Human population growth accompanied by expanded demands for electrical energy has resulted in increased construction of power generating facilities. Conventional fossil fuel power plants are being built to produce many times the electricity of those built in previous years. Nuclear power plants, not economically feasible in small sizes, are being constructed to generate even more electrical energy per unit than the new conventional units. Nuclear power facilities produce heat less efficiently and, thus, require approximately fifty percent more cooling per BTU than usual methods (Kolflat, 1968). Increased exposure of populations and communities of aquatic organisms to thermal pollution can thus be anticipated when natural water systems are utilized as a cooling source.

The majority of research effort relative to the effects of heated waste waters on aquatic systems has been directed towards macro- as opposed to micro-organisms. While the use of microorganisms in general pollution monitoring is not a new concept; for examples see Kolkwitz and Marsson (1908); Butcher (1947); Fjerdingstad (1962), most of the existing effort has been in the area of correlation of species to various polluted situations. The major disadvantages of such approaches as well as the use of populations and communities of microorganisms in pollution monitoring are discussed in Cairns and Lanza (1971) and Cairns et al. (submitted).

Research effort relating to the exposure of aquatic microorganisms to artificial temperature increases has been, in certain instances, concerned with the effects on components of the primary producer level of food chains. The critical ecological significance of such autotrophic organisms is well known.

Diatoms are commonly found as important community components of the periphyton and phytoplankton of major aquatic ecosystems (streams, lakes, and estuaries). The importance of the Chrysophyta, and specifically diatoms as aquatic community components indicating various stream conditions, has been pointed out clearly by Patrick (1949, 1969). Krezoski (1969) demonstrated that diatoms often form the major components of lake phytoplankton communities subjected to both entrainment through steam condensor lines of electric power generating facilities, and overall temperature rises resulting from the addition of heated effluents beyond the outfall. Carpenter (1971) recently demonstrated a dominance of diatoms in phytoplankton communities in studying an estuarine ecosystem. Many estuaries are presently either providing, or being considered as electric power generating facility sites. Of further importance is the fact that diatoms are generally represented by the largest number of species with comparatively low temperature tolerances, i.e. below 30°C.

Some field investigations of the effects of artificial temperature increases on microscopic algae on or near actual electrical power generating units (Dryer and Benson, 1957; Trenbly, 1960, 1965; Warinner and Brehmer, 1965; Beer and Pipes, 1968; Kresoski, 1969;

Morgan and Stross, 1969; Patrick, 1969; Cairns, Kaesler and Patrick, 1970; Jensen, 1970) are available. However, little effort has been put forth relative to controlled laboratory studies designed generally to simulate both passage through steam condenser cooling lines and the introduction of thermal effluents to aquatic ecosystems. The vast majority of accomplished laboratory research has been concerned with comparisons of different temperatures or gradual temperature increases (Dallinger, 1887; Barker, 1935; Margalef, 1954; Wallace, 1955; Cairns, 1956; Anderson and Lommasson, 1958; Sorokin, 1959, 1967; Holton et al., 1964, Kevern and Ball, 1965; Phinney and McIntire, 1965; Rhodes and Herndon, 1967; Patrick, 1968, 1969; Patrick et al., 1969; Kleinschmidt and McMahon, 1970) with little or no effort directed towards abrupt temperature shocks similar to those examined by Ayres (1916), Peary and Castenholz (1964) and Lanza et al. (1970).

The present studies involved both autecological and synecological investigations on diatoms subjected to defined abrupt temperature increases under controlled laboratory conditions. The effects of temperature increases on viable diatom populations and communities isolated from other stress factors, e.g. chlorine, heavy metals, turbulence, pressure, etc. commonly encountered at electric power generating facilities were, thus, ascertained. Experimental design permitted the use of large numbers of physiologically similar cells at various ambient temperatures in two general approaches to assess effects. First, a new technique utilizing fluorescent patterns of

diatom cells was developed and initially tested. The procedure involved the establishment of "normal" patterns of autofluorescence of cellular chlorophyll and accessory carotenoids, and the inducement of lipid fluorescence with 3, 4 Benzpyrene - Caffeine fluorochrome. Deviations from such established "normal" patterns could thus be used to measure major physio-morphological alterations following temperature stress. The technique also provides early detection of sublethal cellular alterations prior to severe disruptions of cellular structure, i.e. as the cell approaches the upper limits of temperature tolerance but prior to total damage encountered beyond such limits. Investigation of the effects of temperature shocks on diatom populations and communities already under multiple stress due to sub-optimal culture (nutrient deficiency, aging, density, etc.) was also carried out.

Secondly, quantitative estimates of the reproduction of diatom populations subjected to some of the same temperature increases were obtained.

MATERIALS AND METHODS

A series of test experiments was conducted prior to the beginning of any studies in order to establish and develop application methodology for the temperature stresses, handling procedures, and microscopic techniques used in these investigations. Autecological investigations consisted of a total of 14 additional experiments covering various stress applications to diatom populations with ambient temperatures of 18, 23, and 29°C.

Several different fluorochromes for various cellular components were considered (Neutral Red, Phosphine 3R, 3,4 Benzpyrene - Caffeine, Acriflavine hydrochloride, etc.) with emphasis upon cellular lipids due to their susceptability to temperature alteration.

Since characterization of lipid in unstained materials is often inconclusive, it was decided to use a lipid fluorochrome. Initial laboratory trials indicated that maximum fluorescence intensity was achieved with 3,4 Benzpyrene - Caffeine (Berg, 1951), a non-differential lipid fluorochrome.

Autecological Studies

All diatom populations were cultured in synthetic water media as described by Cairns (1968). All media was prepared and used within 48 hours. The pH was adjusted to 7.2 with 0.5 N HCL and 0.5 NaOH prior to sterilization in an autoclave. Soil extract used in the media was prepared as described by Barr and Hickman (1967). The same

soil batch was used to prepare all extracts for these experiments. All culture flasks used were Pyrex 250 ml. Erlenmeyer No. 4980 to minimize differences in glass thickness and subsequently the heat retention of flask walls during periods of thermal shock. All glassware was cleaned by the method of Chu (1943). Test flasks were filled with 50 ml. of media (to provide good surface to volume ratio for gas exchange) cotton stoppered, and autoclaved.

Inoculum. Initially the test organisms used were unialgal cultures of Navicula seminulum var. Hustedtii Patr., a moderately sensitive fresh water diatom common to many unpolluted streams. All cultured populations, including stocks used for inoculum,were maintained in Shirer Constant Environment Rooms at controlled temperatures with accuracy to $\pm$ 1°C around each ambient temperature. Constant agitation and aeration was provided by Burrel wrist-action shakers and photoperiod of 16 hours light at an intensity of approximately 2690 - 3228 lux at the culture flask surface was provided by 2 G.E. daylight fluorescent lights. During the remainder of each 24 hour period there was no measurable illumination. Prior to any use in the preparation of inocula, stock populations were maintained in the laboratory for several months with weekly transfers to assure an active division rate characteristic of the species during periods of rapid growth.

Population cell lines at each of three ambient temperatures, i.e. 18, 23, and 29°C, within and spanning the normal optimum growth range of the species were later established. Continuous culture with weekly

transfers for a period exceeding 5 consecutive weeks at each temperature provided actively dividing diatom populations for use as inoculum.

Replicate populations were prepared for various temperature exposures in each experiment by inoculating each flask with 1 ml. of diatoms in uniform suspension to insure uniform numbers. A Waring blender equipped with a sterilized mini-container (110 ml.) was used to insure uniform suspensions of diatoms at a relatively constant temperature. Since blending diatom suspensions generates considerable heat, temperature increases were controlled during this process by packing the exterior base reservoir of the blender mini-container with crushed ice and blending the diatoms for exactly two minutes. Previous tests using this technique demonstrated temperature control at ambient $\pm$ 2°C during blending. This procedure was adhered to throughout all phases of these experiments. Each flask was inoculated with 1 ml. of a uniform diatom suspension. At the time of inoculation, a cell count was made using the A.S.T.M. technique developed by Patrick (1968) to determine the number of diatoms per microscopic field introduced into each culture. A total of 60 fields was counted and statistically analyzed. Since both the cell age and density affect the number of divisions occurring, all inocula used were composed of cells harvested on the seventh day of growth, adjusted to similar starting densities (mean number of cells per microscopic field). Cell counts consisting of 100 fields per population were made at the end of each 7 day experiment and statistically analyzed to estimate the amount of cell division which had occurred in all populations.

Autecological investigations involved duplicate experiments with replicate populations at each of three ambient temperatures, i.e. 18, 23, and 29°C. Experimental design permitted simultaneous inoculation of all populations used in exposure to Stresses 1-3 on day 3 for use in evaluating immediate and delayed effects. Immediate effects of Stress 1 applied on day 7 to 18, 23, and 29°C populations were also measured in separate experiments. Stress 4 was applied in experiments utilizing 29°C populations only.

Temperature Stress. Temperature stress was applied to normal diatom populations in four general categories designed for maximum simplicity and reproducibility. The categories represent certain types of defined abrupt temperature shocks to which aquatic microorganisms might be subjected due to the presence of electric power generating facilities on aquatic ecosystems. The technique used allowed the exposure of intact, actively growing populations at several ambient temperatures within the optimum growth range of the test organism. Temperature stress categories utilized were defined as follows: (1) Stress 1, increases of approximately 10-12°C in less than 20 seconds with a return to approximately ambient in less than 60 seconds; (2) Stress 2, increases of approximately 7°C in less than 1 hour with maintenance at this increase for the duration of the growth period, i.e. approximately 96 hours; (3) Stress 3, a combination of Stresses 1 and 2; and (4) Stress 4, increases of approximately 4°C in less than 1 hour with maintenance at this increase for the duration of the growth period, i.e. approximately 96 hours.

General simulation of certain types of temperature shocks which could possibly result from entrainment through steam condenser cooling lines, and overall downstream addition of thermal effluents were thus attained. All experiments were run for 7 days with stress application within this period. The experimental design measured both immediate and delayed effects of stress on replicate populations with stress application on either day 7 or day 3 respectively. Since cultured diatom populations may exhibit a lag phase up to 48 hours, stress application between days 3 and 7 assured exposure of actively growing replicate cell lines in the exponential growth phase. Also, the third to the seventh day is the time when the growth rate can be most accurately correlated with the effects of the test medium, thus assuring maximum numbers of physiologically similar cells. Temperature stress was applied in two basic ways selected for their simplicity and control. The first, Stress 1, involved flasks fitted with sterile thermometer equipped stoppers for visual temperature monitoring. Stress 1 was effected by immersing the flask containing an intact diatom population into a 100°C water bath for exactly 8 seconds. Removal of the flask allowed a continued temperature rise due to the heat retention of the glass until 9-10°C above ambient was attained. The flask was immediately immersed into a cold water bath at 5°C for 20 seconds and removed. The temperature continued to rise 1-2°C during this immersion, and then leveled off beginning the descent to approximately ambient which occurred in less than 60 seconds. Stress 2 was attained by simply transferring intact diatom populations to a

second environmental growth chamber maintained at 7°C above the ambient temperature under test. Such a transfer permitted the temperature of the intact population to reach the 7°C increase in less than 1 hour. Stress 3 involved exposure of populations to a combination of both Stress 1 and 2, just described above. The last category, Stress 4, was added to provide information relative to the 29°C ambient populations (upper growth range) only. It became evident in the preceding experiments that these populations experienced severe damage following Stress 2. As a result, experiments involving a lower but equally abrupt temperature stress, Stress 4, were carried out. The purpose of these experiments was to measure any sub-lethal transitional effects occurring prior to severe destruction of 29°C populations following Stress 2. Stress 4 was effected by transferring 29°C populations to another environmental growth chamber maintained at 4°C above ambient.

In addition, experiments were conducted on older, less viable diatom populations under prolonged culture at 20°C in the same media, i.e. 14-30 days. These studies involved two organisms, Navicula seminulum var. Hustedtii Pat. and N. pelliculosa (Breb.) Hilse. Fogg (1959) suggested in discussing laboratory studies of diatoms and other algae that fat accumulated in nitrogen deficient cultures. Prolonged culture in the same media with high density populations was carried out here to produce large numbers of diatoms under multiple stress, including nutrient deficiency, crowding, etc. These diatom populations were investigated as to (1) changes in their "normal" fluorescent patterns due in part to increased fat resulting from such stress, and

(2) changes following additional stress from temperature shocks. Temperature stresses in these experiments were administered in controlled temperature water baths as increases above ambient of (1) 60°C for 2 hours, (2) 20°C for 24 hours, and (3) 10°C for 24 hours. The extremely abrupt temperature shock of 60°C for 2 hours was designed to induce extreme cellular alterations as a reference point in appraising lower, more realistic temperature shocks. The effects of the various stress categories were examined relative to immediate and delayed responses of diatom populations. Stress 1 was applied on days 3 and 7 in different experiments. Stresses 2-4 were always applied on day 3. All population sampling for examination of effects was done on day 7.

Synecological Studies

Collection and Culture. Synecological studies were conducted to assure positive correlations between cellular fluorescence patterns of laboratory populations and dominant species components of mixed natural field communities. Various different ponds and streams were initially sampled for suitable test material. However, all community collections used in test cultures in the described experiments were collected in the New River in Giles County, Virginia. Since diatoms are usually a dominant group in the fall, spring, and winter, samples were taken from diatometers (Patrick, Hohn, and Wallace 1954) at appropriate times in the winter and spring months at various existing ambient temperatures. Diatometer slides were placed in the river to permit colonization by existing species for 7-21 day periods at the same

collection site for use in various experiments. Once colonized all collections were returned to the laboratory immediately and prepared for continuous flow and static culture maintenance. Water chemistry consisting of dissolved oxygen, alkalinity (as $CaCo_3$) total hardness, carbon dioxide, pH, and temperature were taken at collection time using a HACH model AC-36-WR water testing kit.* While such water chemistry does not provide definative evidence, it supplies general information about the environmental conditions under which the diatoms were collected.

Continuous flow culture provided a reliable means of in lab maintenance of intact diatom communities on the original field collection substrate. This permitted a study of the normal fluorescent patterns of dominant community components. Such patterns could then be compared to those seen in populations under normal autecological culture. In addition some limited studies of the immediate effects of one type of short-term defined temperature shock could be evaluated. The continuous flow culture system consisted of maintaining colonized slides exposed to a continuous flow of water of the same general physio-chemical characteristics of the collection site. Communities were kept in artificial plexiglass streams in a laboratory ecosystem described previously in detail in Lorton and Cairns (1971). The reservoir tank of the continuous flow laboratory ecosystem contained circulating dechlorinated New River water. Additional water chemistry was carried

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*Hach Chemical Co., Ames, Iowa 50010 U.S.A.

out in the laboratory ecosystem and compared to the field site on each collection day to confirm physio-chemical similarity. Flow rates and temperature from each culture trough in use were checked daily and recorded.

Static cultures were designed to expose natural diatom communities to conditions generally similar to the prolonged, high density cultures previously described in the autecological studies. The general effects of temperature shocks as an added stress on these communities relative to light and ultraviolet microscopic surveys could, thus, be compared to those seen in autecological studies.

Static cultures were prepared as follows. The contents of diatometer slides were scraped into water in which they were collected, passed quickly through nylon bolting cloth to remove extraneous material and poured into 16 1/2 x 10 1/8 x 2 1/4 enamel pans filled with water from the collection site. Static cultures were stored in Shirer Environmental Chambers to acclimate communities to the laboratory ambient temperature of 20°C ($\pm$ 1°C). A photoperiod of 16 hours light (at an intensity of 2690 lux at surface of the culture) and 8 hours with no measurable illumination per 24 hours was also maintained. Cultures were maintained for a maximum of 7 days; older cultures were discarded.

Temperature Stress. Numerous fluorescent surveys on dominant community components consisting of normal diatom communities and those from sub-optimal cultures were carried out to establish characteristic cellular patterns prior to their use in any experiments. Normal diatom

communities examined came from two sources; (1) samples collected from the river site and immediately returned to the lab and placed on test, and (2) samples maintained in the lab under continuous flow culture.

Because of the difficulty of simulating temperature shocks of extended duration under continuous flow conditions, only abrupt, extremely short term types could be effected. The purpose of these limited studies was to try and detect immediate and short term delayed effects. Long term effects were not considered. Here, immediate temperature increases of 16-18°C for less than 30 seconds with immediate return to ambient were accomplished as follows. Diatometer slides (16 total) seeded with a 7 day old community were collected and transferred to the laboratory. In the lab, the slide communities were maintained under continuous flow as previously described for an additional 7 days for generally acclimation prior to testing. The temperatures at the river collecting site and the lab ecosystem were identical, i.e. 22°C. Two troughs served as controls while another two were exposed to increased temperature. Temperature stress was applied by pumping heated water from the artificial ecosystem's head tank into the two experimental area troughs until the temperature stabilized at 18-21°C. Four slides from the holding trough at 22°-23°C were quickly transferred to each of the experimental troughs and left for 25 seconds. The slides were then quickly returned to the control trough and continued in culture at ambient. Four control slides were handled similarly by transfer from troughs at 22°-23°C to different

troughs at the same temperature. Samples consisting of 2 slides were taken from each trough at 1 and 24 hours following shock and examined for immediate and delayed effects respectively. Community components were scraped from slides into beakers of distilled water and wet preparations were made from the beaker contents as needed for microscopic surveys. The surveys consisted of examining 1000 diatoms at random and noting the general cellular patterns of the majority of community components. The remainder of the beaker contents was preserved in 4% formalin for later identification of dominant community components.

Static community cultures were exposed to the same temperature stresses described previously for prolonged culture, high density population studies. Test cultures were prepared by taking 50 ml. aliquots of diatom suspensions from stock pan cultures and dispensing them into 250 ml. cotton stoppered Erlenmeyer test flasks on the day the experiment commenced. Counts were done in a Sedwick Rafter Chamber to estimate the number of diatoms per ml. in the inoculum. Replicate communities were run in each treatment group. Experimental communities growing at 20°C were placed in water baths at the various elevated temperatures for the indicated time periods. Control communities were also kept in water baths at ambient temperatures during experimental periods. At the conclusion of the temperature stress periods, each culture was sampled and examined. Surveys were carried out in the same fashion described for the continuous flow communities.

Examination Criteria

A total of 36 experiments was conducted to determine the feasibility of utilizing the following criteria in measuring changes in diatoms resulting from exposure to defined abrupt temperature shocks: (1) General surveys carried out under the light microscope to note major changes in total cellular content, pigmentation, and general structure; (2) Autofluorescence surveys to note major physio-morphological alterations in the cell metalloporphyrin content (Chlorophyll a + c) as expressed by chloroplast content and ultraviolet fluorescence. Thermal damage to the labile carotenoid accessory pigments, characteristic of the Chrysophyta, would also undoubtedly be manifested through the cell's metalloporphyrin fluorescence patterns; (3) Induced fluorescence surveys on total visible cellular lipid patterns using 3,4 Benzpyrene - Caffeine lipid fluorochrome; (4) The ability of diatoms to survive and reproduce (autecological studies only).

Fluorochroming and Fluorescent Surveys

All diatom samples were taken from a uniform suspension immediately following mixing in a Waring mini-container blender packed with ice as described previously. Cells were concentrated by centrifugation at approximately 3100 r.p.m. in 15 ml. conical centrifuge tubes for 2 minutes. After removal of the supernatant media, cells were fluorochromed by the method of Berg (1951) with 3,4 Benzpyrene complexed to Caffeine in aqueous solution. Cells remained in the fluorochrome for

20 minutes followed by re-concentration by centrifugation after which supernatant fluorochrome was removed. Cells were given three successive rinses by dilution through re-suspension in sterile distilled water. After re-concentration by centrifugation, cells were immediately examined to assure maximum fluorescence. Samples were also prepared lacking the fluorochrome to serve as background fluorescence controls. Wet preparations were made on standard 3 x 1" microscope slides rinsed in distilled water with 24 x 50 mm. coverslips and scanned with a random recording of fluorescence patterns. A total of 1000 cells from an aliquot representing approximately one third of the total culture population or community were examined.

In autecological experiments involving normal N. seminulum cells (Experiments 1-14) results were recorded as to the specific percent displaying altered fluorescent patterns. In all synecological and sub-optimal culture studies the major fluorescent trends noted in 1000 cell surveys were recorded. Altered cells represented any readily visible deviation from the established normal patterns of autofluorescence and induced fluorescence. These were described accordingly in different shock categories.

Ultraviolet microscopic surveys were conducted on a Leitz Ortholux microscope equipped with an ultraviolet light source from an HBO 200 mercury lamp with BG 38 and UG 1 filters. Additional surveys on chlorophyll autofluorescence were also conducted with BG 38 and BG 12 filters.

RESULTS AND DISCUSSION

All results presented here are based on the application of the previously described examination criteria on autecological and synecological experiments. There are several difficulties in identifying and evaluating algal cellular physiological alterations prior to or after cell expiration utilizing light microscopic surveys. Severe, consistent morphological damage afflicting the majority of species is usually accompanied by cell expiration and offers limited assistance in detecting sub-lethal cellular abberations. Since only certain species of diatoms exhibit motility, movement as a criterion for cellular damage is generally inadequate. Reproduction as a manifestation of growth, and primary productivity have been used with some success in partial evaluations of various pollutional stresses.

Light microscopic examinations to provide reference data on control diatoms from regular autecological and continuous flow synecological culture revealed similar general cellular characteristics. Normal cellular content, pigmentation, and intact structure prevailed in the majority of diatoms. Light microscopic appraisals of control sub-optimal cultures, i.e., prolonged high density autecological and static synecological cultures, revealed certain general differences. Cellular content and pigmentation were somewhat reduced in many dominant community components of the control static synecological cultures. Autecological cultures while displaying little visible decrease in total content and pigmentation did appear more opaque with many cells displaying

deformed structures usually manifested by flattened or blunt cell ends.

Attempts were made here to develop and evaluate the use of new criteria, i.e., total cellular fluorescent patterns with emphasis on lipids and metalloporphyrins, as a measurement of lethal and sub-lethal cellular alterations following stress. Hopefully the data generated here can provide insight as to major physio-morphological alterations resulting from the exposure of diatoms to abrupt thermal stress.

Initial ultraviolet microscopic surveys on control diatoms established normal cellular fluorescent patterns under various culture conditions for reference purposes. Comparison of control cells from regular autecological and continuous flow synecological culture revealed great similarity. Control diatoms were consistently dominated by a characteristic red autofluorescence of the cell chloroplast. Accompanying this was limited faint blue 3,4 Benzpyrene - Caffeine induced lipid fluorescence. The majority of lipid fluorescence surrounded the chloroplast in close proximity to cellular poriferal membranes and represented a minute percentage of the total cellular fluorescence. Additional blue to blue-white lipid as a "grainy" scattered pattern within the red chloroplast tended to impart a red to red-pink (blue-white + red) fluorescence to the chloroplast. The apparent lack of visible cell lipid after fluorochroming is in agreement with normal log phase physiology; i.e. cells in the exponential growth phase possess high quantities of protein, chlorophyll, and nucleic acids and low quantities of carbohydrate and fat. In other

studies, Navicula pelliculosa following photosynthesis for 2 minutes in the presence of C^{14} -labeled bicarbonate demonstrated less than 20 percent entering the fat fraction (Fogg, 1966). While control cells cultured at 18, 23, and 29°C were generally similar in gross fluorescent characteristics, 29°C diatoms indicated a possible slight decrease in chloroplast content.

Surveys of control cells from populations under sub-optimal culture, i.e. prolonged, high density autecological and static synecological culture, also revealed greatly similar fluorescent patterns when compared. In addition, major deviations from the patterns just described for cells under more optimum culture were clearly evident. Marked increases in visible cellular lipid fluorescence were seen in the vast majority of diatoms observed. One noticeable consistent exception was Diatoma vulgare Bory. var. Breve Grun which often lacked such visible increase in induced lipid fluorescence. In most other species, however, high concentrations as blue-white spherical deposits on either side of the visible central chloroplast were characteristically present. The increased lipid probably can be attributed to cell degeneration resulting in increased lipid metabolism as described by Fogg (1966).

Autecological Studies

Experiments 1 and 2 (Table I) involved the exposure of replicate diatom populations at an ambient temperature of 18°C to Stresses 1-3 on day 3 of the growth period. Light microscopic surveys demonstrated no consistent differences between any control or experimental populations with reference to cellular content, pigmentation, and general

cellular structure. Ultraviolet fluorescence microscopic surveys also failed to demonstrate any consistent differences between control and experimental populations. The vast majority of cells displayed patterns characteristic of viable log growth phase diatoms. Of the very few altered cells seen in all populations, the majority possessed increased lipid. A great many of these could be the original cells introduced as inoculum at the beginning of the growth period. Reproduction data relative to all replicate populations in experiments 1 and 2 (Fig. 1,2) indicated no consistent decreases resulting from exposure to Stresses 1-3. Reproduction was expressed as an estimate of the number of divisions occurring in the 7 day growth period. However, the possibility of some irregular growth stimulation in certain populations following Stress 2 was evident. This is probably best explained by the fact that the populations were moved closer to their optimum growth temperature by the 7°C increase without any noticeable effects of the abruptness of the increase (less than 1 hour). Patrick (1969) has demonstrated similar stimulation with gradual temperature increases on components of glass slide diatom communities in controlled greenhouse studies. Here, increasing temperatures over the naturally occurring temperature resulted in biomass increases until temperatures of 29-30°C were attained.

Experiments 3 and 4 (Table II) were designed to investigate the immediate effects of Stress 1 on day 7 of the growth period relative to cell physio-morphology. Both light and ultraviolet microscope surveys failed to indicate any differences between control and

experimental populations following stress.

Populations midway in the growth range at an ambient temperature of 23°C were investigated following Stresses 1-3 on day 3 in experiments 5 and 6. All populations examined did not contain any consistent differences under all microscopic surveys. Table III summarizes data from the fluorescent surveys and displays extremely small numbers of cells with physio-morphological alterations. The few cells with deviated fluorescent patterns were like those described for 18°C populations.

Population growth data for 23°C ambient diatoms is summarized in figures 3, 4. Here, similar estimates of reproduction expressed as number of divisions can be seen between all populations following all stresses. Experiments 7 and 8 (Table IV) involved a study of the immediate effects on 23°C populations exposed to Stress 1 on day 7. No significant differences in visibly altered diatoms relative to fluorescent surveys were noted.

Experiments 9 and 10 involved studies of diatoms acclimated near the upper threshold of the optimum growth range of the species, i.e., 29°C. Here (Table V) exposure of populations of N. seminulum to Stress 1 produced no significant differences, immediate or delayed, in the total percent of altered cells seen in all microscopic surveys. Only normal numbers of cells with altered fluorescent patterns were noted. Exposure to Stresses 2 and 3, however, produced a marked effect on all diatom populations tested. The vast majority of stressed cells viewed had major cellular alterations. Light microscopic comparisons

of experimental and control populations revealed reductions in cellular content and the appearance of internal opaque areas in many experimental cells. The pigmentation of the majority of stressed cells was characterized by a green coloration as opposed to the yellow-brown coloration characteristic of normal diatoms. This striking brown to green color change undoubtedly was the result of temperature induced destruction of the labile carotenoids characteristic of diatoms as a group.

Fluorescent surveys aided in better defining the physio-morphological damage seen under the light microscope. Stressed cells revealed various lipid fluorescent patterns ranging from increases like those seen in the few altered cells of 18 and 23°C populations, to decreases causing cells to be barely visible as faint blue "ghosts". Autofluorescence surveys of chlorophyll in cells following Stresses 2 and 3 also demonstrated marked differences from control diatoms. A total lack of the characteristic red fluorescence was seen indicating degradation of the cellular chlorophyll content in most of the populations.

Experiments 11 and 12 (Table VI) involved exposing 29°C populations to Stress 1 on day 7 to measure any immediate effects. Light and ultraviolet microscopic surveys failed to detect increased numbers of cellular alterations following the standard criteria.

Growth studies of populations acclimated to an ambient temperature of 29°C are summarized in Fig. 5, 6. Exposure of diatoms to all categories of stress produced negative effects upon population growth. Stress 1 alone, which had no measurable effect upon the majority of diatoms acclimated at 18 and 23°C, caused a decrease in division in

most upper threshold populations as shown in data estimating final population numbers. Here, an extremely abrupt temperature shock of very short duration, i.e. 10-12°C increases above ambient in less than 20 seconds with a return to ambient in less than 60 seconds, produced results of possible considerable ecological significance. Temperature shocks of this general type alone and accompanied by other shocks can be anticipated in new high volume cooling systems of power generating facilities. Stresses 2 and 3 also produced population reductions, but of a more severe nature than Stress 1 alone. Stress 2 alone and Stress 3 (Stress 1 + 2) produced the same general results in decreased divisions. The overall results of upper threshold populations exposed to abrupt temperature stress can be summarized as follows: (1) a general decrease in the number of divisions occurring in the normal growth period resulting in a lower number of individuals following all stresses, and (2) severe physio-morphological damage to the diatoms produced during decreased division following Stresses 2 and 3.

The dramatic shift in total cellular fluorescent patterns in 29°C populations following Stresses 2 and 3 prompted further experiments. These were designed to demonstrate the possible existence of a transitional phase of stress induced physio-morphological alterations occurring at a lower abrupt temperature increase. Stress 4 was designed to fill this need and provided a 4°C increase in less than 1 hour.

Experiments 13 and 14 (Table VII) involved exposing 29°C populations to Stress 4 alone on day 3. Light microscopic surveys indicated no

general decreases in total cell content, or consistent, easily noted structural changes. However, opaque areas and some reduction in the intensity of pigmentation predominated in many of the cells examined. Interestingly, general total cellular fluorescent patterns of the vast majority of these stressed cells were like those seen and described for diatoms stressed in sub-optimal culture prior to any temperature shock. Markedly increased lipid fluorescence (3,4 Benzpyrene - Caffeine induced) accompanied by decreased visible red chlorophyll autofluorescence was the common pattern.

The results of experiments 13 and 14 indicate a transitional or intermediate pattern in total cellular fluorescence lying between diatoms growing in log phase at 29°C with no stress and those receiving severe damage following Stress 2 or 3. As noted previously, the vast majority of cells growing at 29°C possess a dominance of red chlorophyll autofluorescence with little, faint blue induced lipid (3,4 Benzpyrene - Caffeine) fluorescence. Following Stress 4 (abrupt 4°C increase), a change in pattern to decreased red chlorophyll autofluorescence and increased blue to blue-white induced lipid fluorescence occurs. A greater total increase, i.e. Stress 2 (7°C increase), or Stress 3 (Stress 1 + Stress 2) results in a general decrease in total cellular fluorescence; both red chlorophyll autofluorescence and blue-white induced lipid fluorescence.

Figures 5 and 6 summarize growth studies of 29°C acclimated populations following Stress 4. Here, a lower number of divisions was noted at the end of the growth period.

The differences of up to approximately one division evident between certain control populations in separate experiments at 18°C (Experiments 1 and 2) and 29°C (Experiments 9, 10, 13, 14) were found to be normal for the culture system employed. Many factors inherent in the technique may be considered in explaining such differences as the variability in the duration of the lag phase, or slightly irregular division due to the close proximity of the ambient temperatures to both extremes of the growth range.

Experiments 15-24 were designed to test different temperature shocks upon diatom populations already revealing stress resulting from sub-optimal culture. Cells from prolonged high density autecological culture at 20°C bearing increased lipid content were studied for major deviations from normal patterns. Experiments 15 and 16 involved exposures of N. pelliculosa to 60°C increases for 2 hours followed by immediate microscopic surveys. Experiments 17 and 18 involved the same treatment with N. seminulum. Light microscopic examinations of all populations revealed severe cellular damage in most cases with structural alterations of cell contents (coagulation) and destruction of normal pigmentation patterns; i.e. the characteristic brown-yellow color of the cells was changed to green. Ultraviolet microscopic examinations indicated that a large part of the internal cellular damage was manifested by a decrease in visible cellular lipid and subsequent induced fluorescence. Varying degrees of decreased red chlorophyll autofluorescence were also seen. Similar results were seen in experiments 19-22 where 20°C prestressed populations of N.

seminulum (Experiments 19, 20) and N. pelliculosa (Experiments 21, 22) were exposed to increases of 20°C (40°C) for 24 hours. The major visible differences between the two shocks under the ultraviolet microscope was more variable damage in experiments 19-22 than 15-18. Generally, a range in degrees of fluorescence from typical control patterns to an almost complete lack of fluorescence was characteristic of cells stressed in experiments 19-22 while more severe, consistent decreased fluorescence was noted in experiments 15-18. Populations exposed to temperature shocks of 10°C above ambient for 24 hours were also observed in experiments 23 and 24 using populations of N. seminulum. No consistent major differences between controls and experimentals were noted in these surveys.

Synecological Studies

Experiments 25 and 26 were carried out on communities collected in the field at 20°C and immediately returned to the laboratory for studies of normal fluorescent patterns and those occurring after temperature stress. Table VIII lists the dominant species characterizing their communities. Control cell patterns under the light and ultraviolet microscopes were like those described for the normal diatoms from autecological culture. Here, exposure to increases of 20°C for 24 hours produced overall decreases in total cellular fluorescence also similar to those noted in the autecological studies.

Dominant components of diatom communities in continuous flow culture experiments are listed in Table IX. In these limited investigations

emphasis was directed towards establishing normal cellular patterns for comparison to laboratory cultured autecological diatoms. Some effort went towards a partial appraisal of one type of temperature stress. Experiments 27 and 28 involved the exposure of communities growing at 22-24°C to abrupt temperature elevations of 16-18°C for 30 seconds. Dominant species were surveyed immediately after shock for visible effects upon cellular patterns only. No reproductive or delayed physio-morphological effects manifesting beyond the short 24 hour study period were carried out. General comparisons were made to the effects of Stress 1 on day 7 as applied to N. seminulum in autecological culture at 23°C. Examinations of 1000 cells selected randomly of the various species in these communities like those in the N. seminulum studies did not reveal significant differences between control and stressed groups. It should be noted that examinations to characterize the structure of the communities used in these experiments indicated a predominance of Gomphonema sp., certain species of which are known to be broadly temperature tolerant (Wallace, 1955, Patrick, 1969).

Experiments 29-36 involved evaluating the effects of temperature stresses on static synecological cultures collected during the winter and early spring at various ambient temperatures. Stresses were the same as those previously described for sub-optimal autecological studies. As in previous efforts, all experiments were repeated for confirmation at different times with similar communities from the same collection. Dominant components characterizing the community structure of cultures used in experiments 29-34 are listed in Table X.

Experiments 29 and 30, 31 and 32, 33 and 34 received increases of 60°C for 2 hours and 20 and 10°C increases for 24 hours respectively. Immediate microscopic surveys revealed results generally similar to those discussed for the sub-optimal autecological studies with the exception of the 10°C increase. The majority of dominant community components receiving 60 and 20°C stresses displayed severe cellular damage at the conclusion of the stress period. A degradation of cellular content and pigmentation resulting in the brown-yellow to green color transformation previously noted along with a general overall decrease of cellular lipid and chlorophyll fluorescence were evident. Communities subjected to the 10°C increase, however, had a general decrease in cellular fluorescence which was not evident in the autecological studies. This may be due to the fact that the communities were originally collected at an ambient temperature of 9-10°C prior to pre-test lab acclimation at 20°C. While acclimation in the laboratory at a 10°C increase above the natural site ambient could be tolerated by the dominant community components, the additional test increase of 10°C was undoubtedly in excess of their upper threshold. This view is supported by later studies, experiments 35 and 36, using communities (Table XI) collected at the same site at an ambient temperature of 15-16°C and acclimated to 20°C prior to testing. Here, temperature stresses of 10°C for 24 hours produced results similar to those seen in autecological studies; i.e. no consistent significant differences were noted.

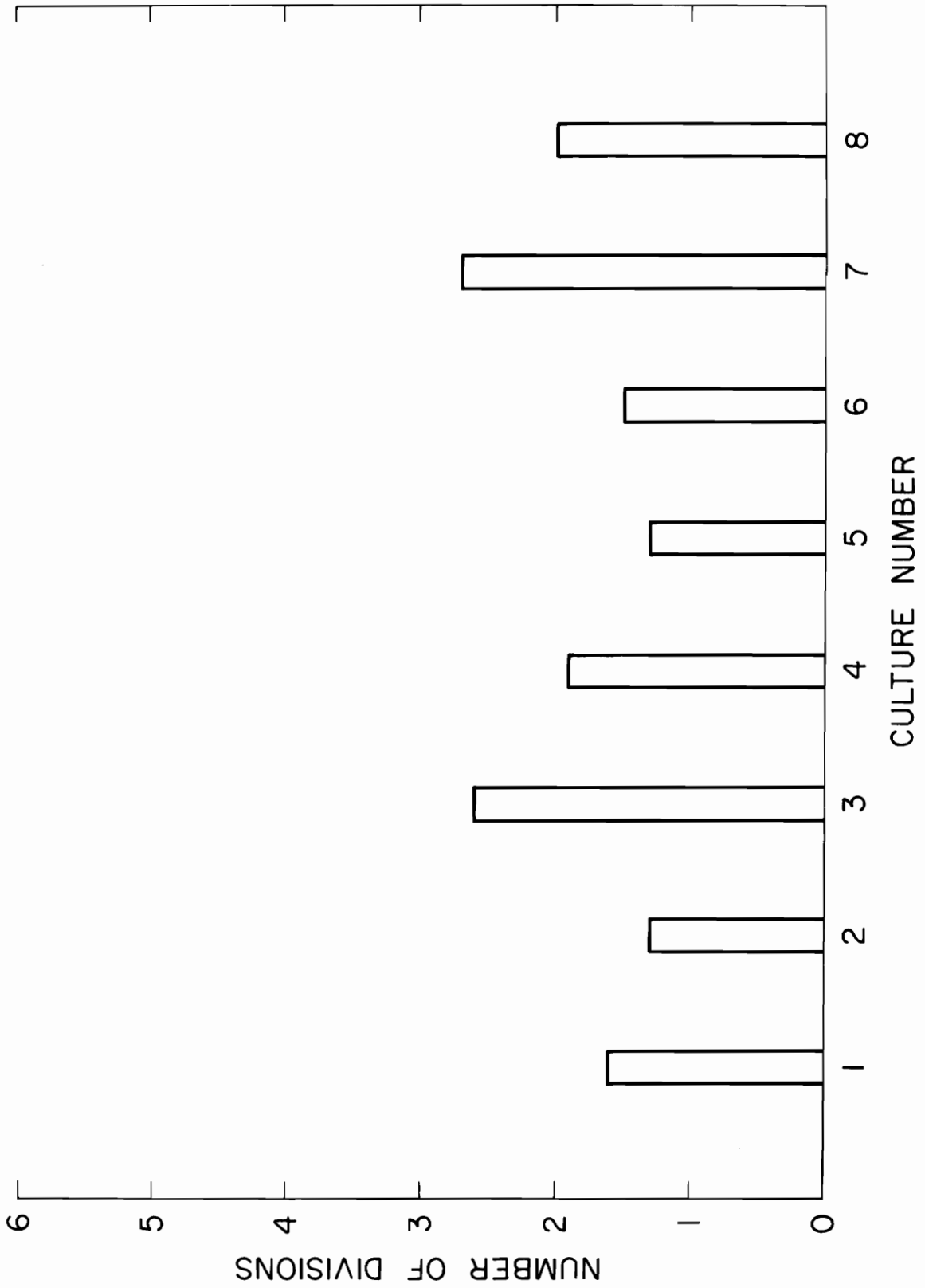
Several general ecological and physiological implications can be

noted in the results of these studies. First, a substantial amount of the intracellular lipid or its degradation products could be exuded out into the surrounding environment following the disruption of cell membranes by certain temperature stresses. The decrease in visible lipid seen here could be, at least in part, explained by such losses. If so, one general result would be an increase in lipid macromolecules in the environment, especially in the surface microlayer. Secondly, the nutritive value of diatoms would be considerably altered by any major change in its lipid content and this could have profound effects on other components of the food web. Thirdly, critical physiological relationships would obviously be affected by sub-lethal alterations in cell lipid. For example, it has been demonstrated that oil droplets normally accumulate in diatoms at the time for division approaches, and disappear following mitosis (Patrick and Reimer, 1966). Also, vertical migration of some species of algae, including diatoms, is dependent upon specific gravity changes due, in part, to cell oil globules (Hoar, 1966). Finally, damages to cell metalloporphyrin and labile carotenoid accessory pigments are of obvious significance in depressing autotrophic metabolism.

The difficulty in attempting to explain natural population and community dynamics from the results of laboratory autecological and synecological studies are acknowledged. It should be emphasized that the ultimate effects of actual heated waste water discharges upon a receiving system should be a major factor in deciding whether such discharges are deleterious. Although some of the changes noted

might be harmful to the aquatic community in a receiving system, final evidence obtained from the receiving system itself will be necessary before one can state with assurance whether they do in fact occur. The value of work of this type is to isolate and indicate some of the factors which should be examined in the aquatic communities of systems receiving heated waste water discharges. Large scale simulation units which encompass more of the complex cause-effect pathways than usual types of laboratory experiments must also be utilized.

Figure 1. Summary of reproduction data for 18°C ambient populations of *N. seminulum*. Experiment 1. (Culture 1,2 = control, no stress; Culture 3,4 = stress 2; Culture 5,6 = stress 1; Culture 7,8 = stress 3).


NUMBER OF DIVISIONS
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1
2
3
4
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6
CULTURE NUMBER
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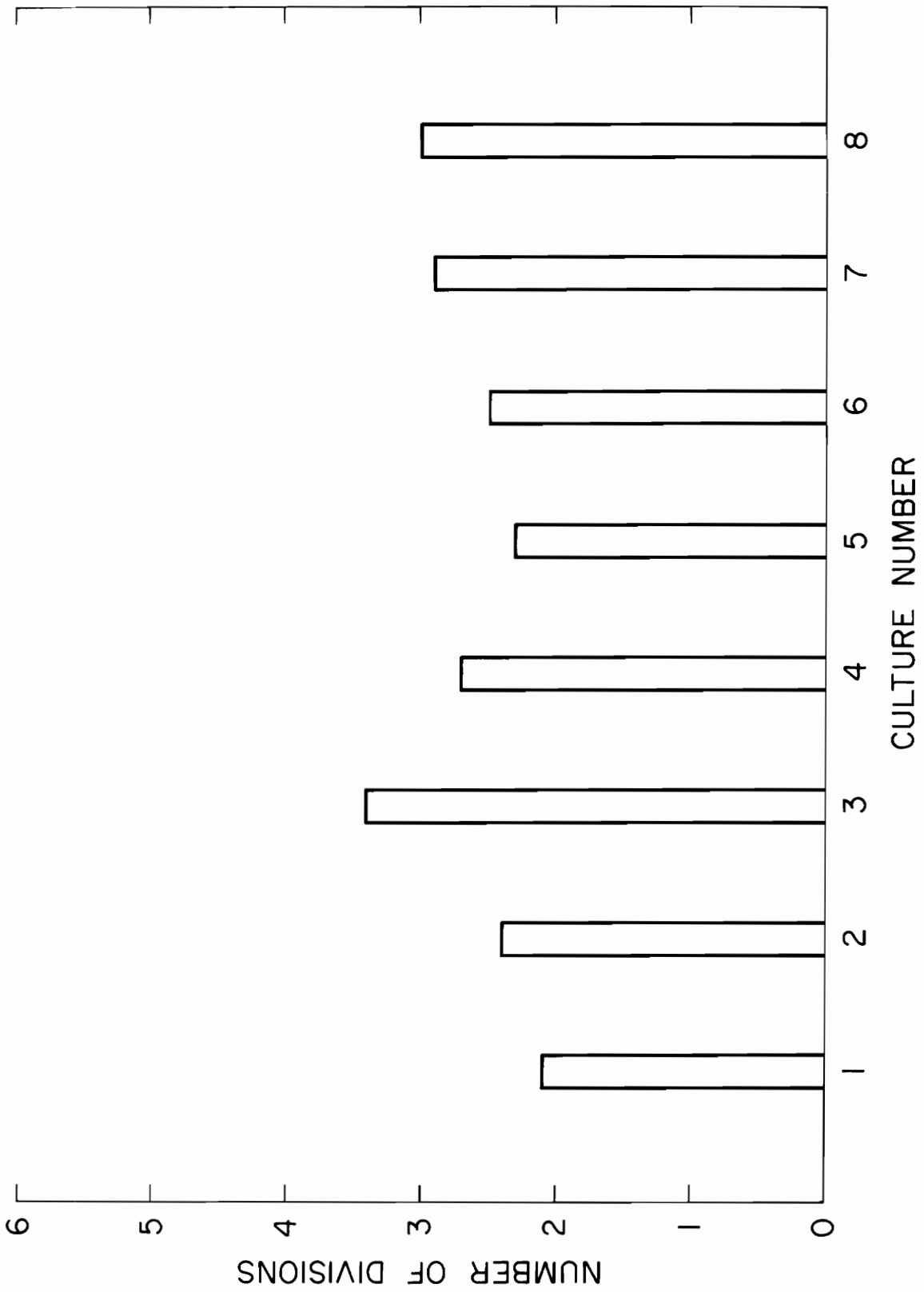
Figure 2. Summary of reproduction data for 18°C ambient populations of N. seminulum. Experiment 2. (Culture 1,2 = control, no stress; Culture 3,4 = stress 2; Culture 5,6 = stress 1; Culture 7,8 = stress 3).


NUMBER OF DIVISIONS
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CULTURE NUMBER
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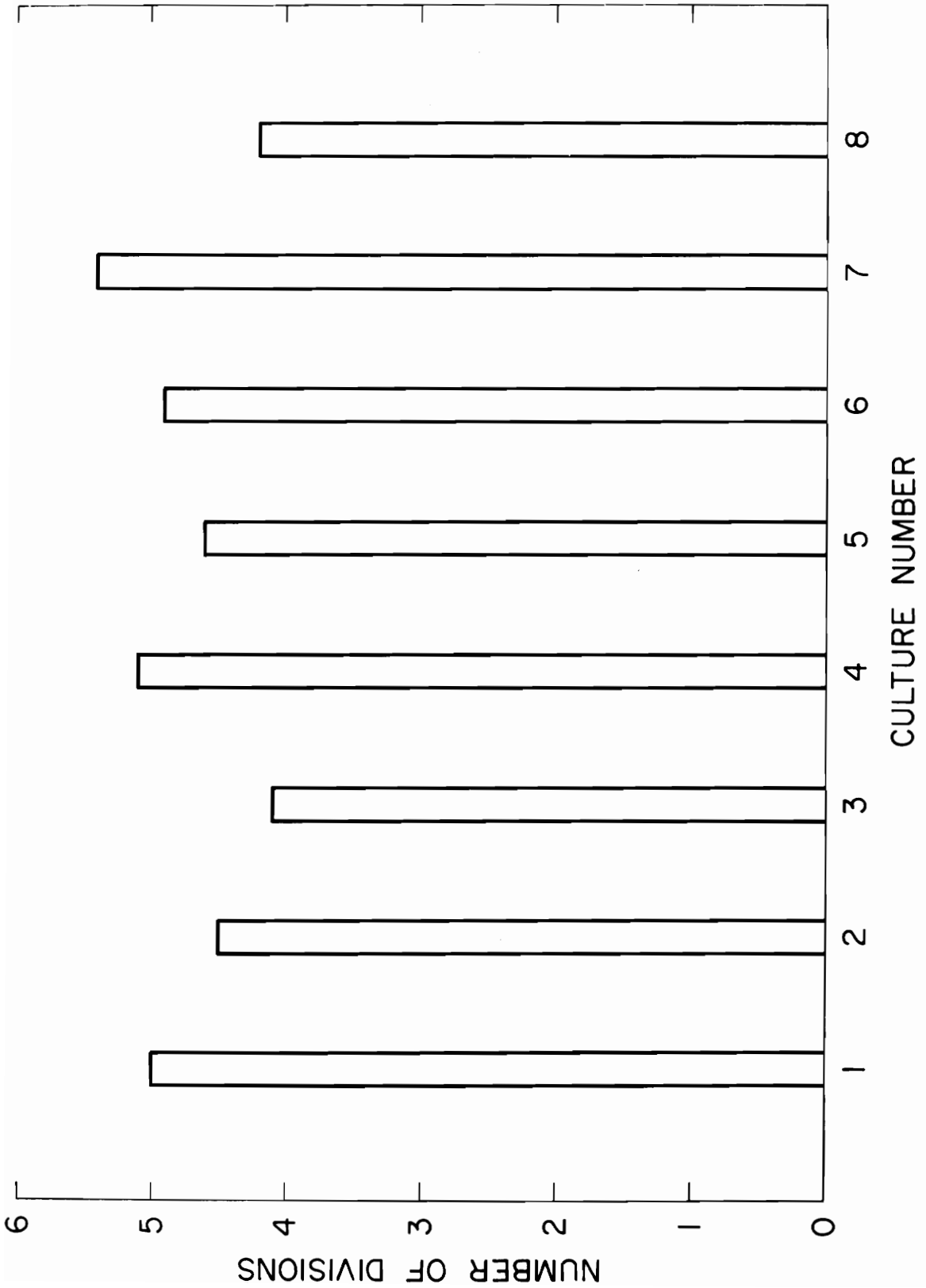
Figure 3. Summary of reproduction data for 23°C ambient populations of N. seminulum. Experiment 5. (Culture 1,2 = control, no stress; Culture 3,4 = stress 2; Culture 5,6 = stress 1; Culture 7,8 = stress 3).


NUMBER OF DIVISIONS
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CULTURE NUMBER
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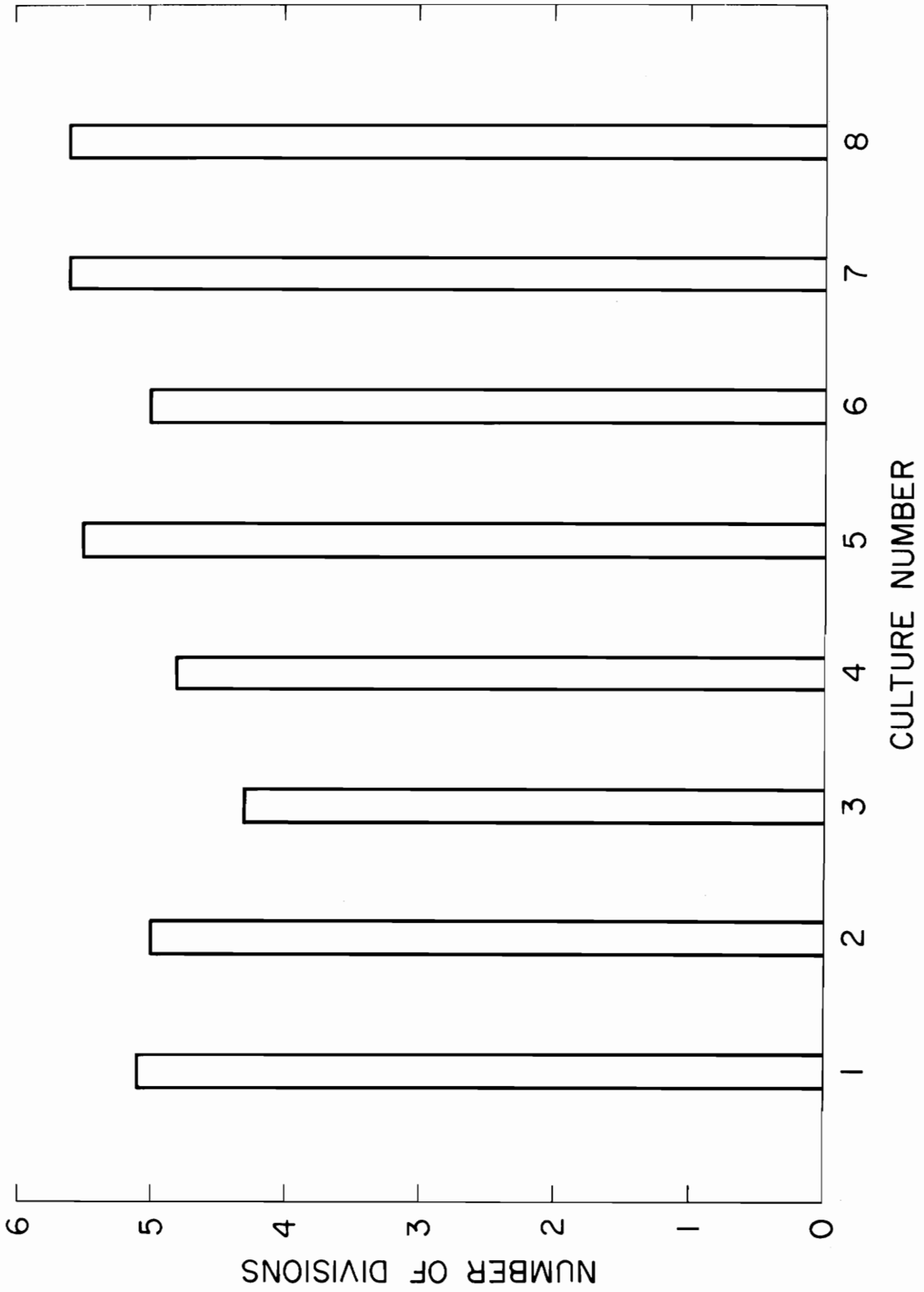
Figure 4. Summary of reproduction data for 23°C ambient populations of *N. seminulum*. Experiment 6. (Culture 1,2 = control; no stress; Culture 3,4 = stress 2; Culture 5,6 = stress 1; Culture 7,8 = stress 3).


NUMBER OF DIVISIONS
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CULTURE NUMBER
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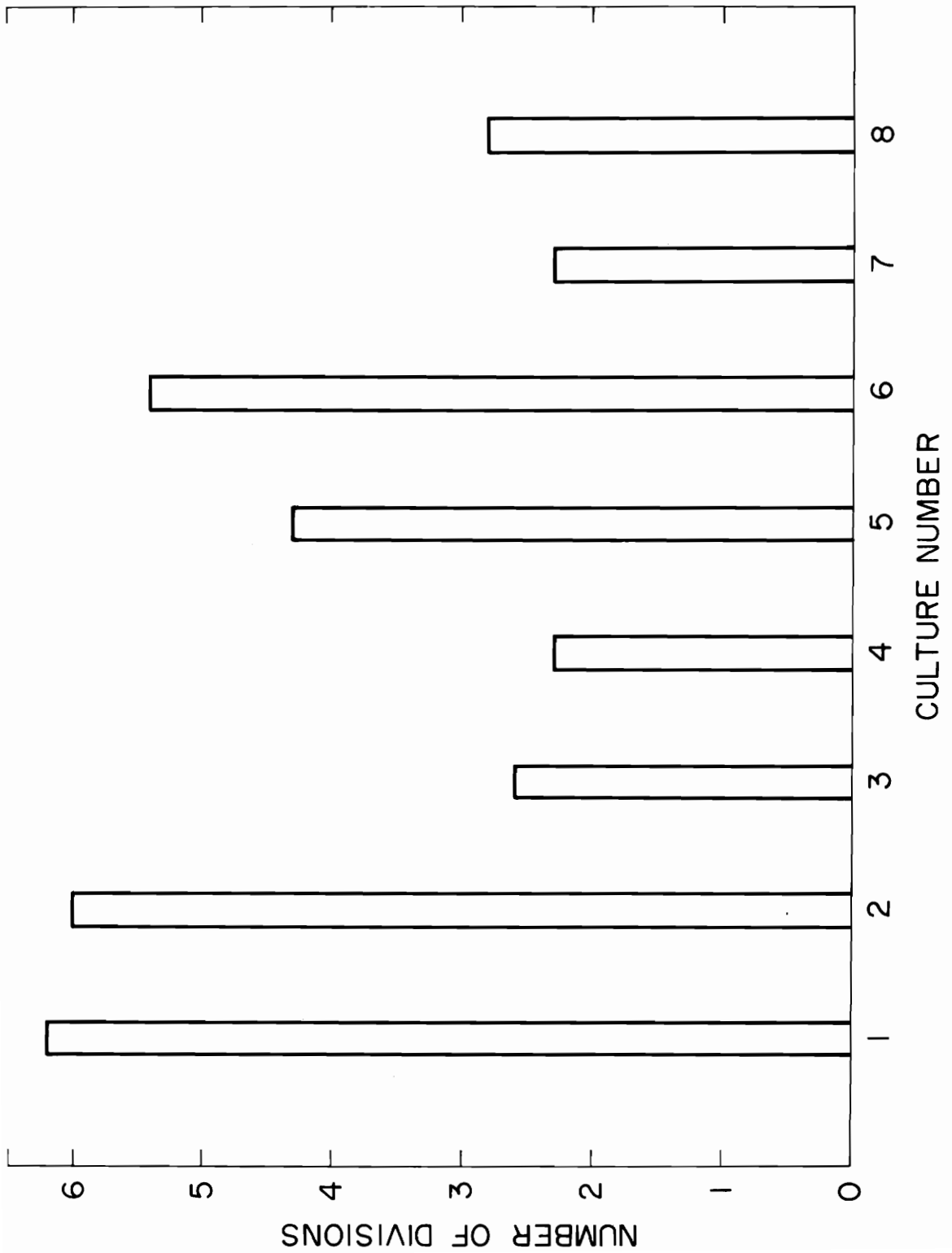
Figure 5. Summary of reproduction data for 29°C ambient populations of *N. seminulum*. Experiment 9. (Culture 1,2 = control, no stress; Culture 3,4 = stress 2; Culture 5,6 = stress 1; Culture 7,8 = stress 3).


NUMBER OF DIVISIONS
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CULTURE NUMBER
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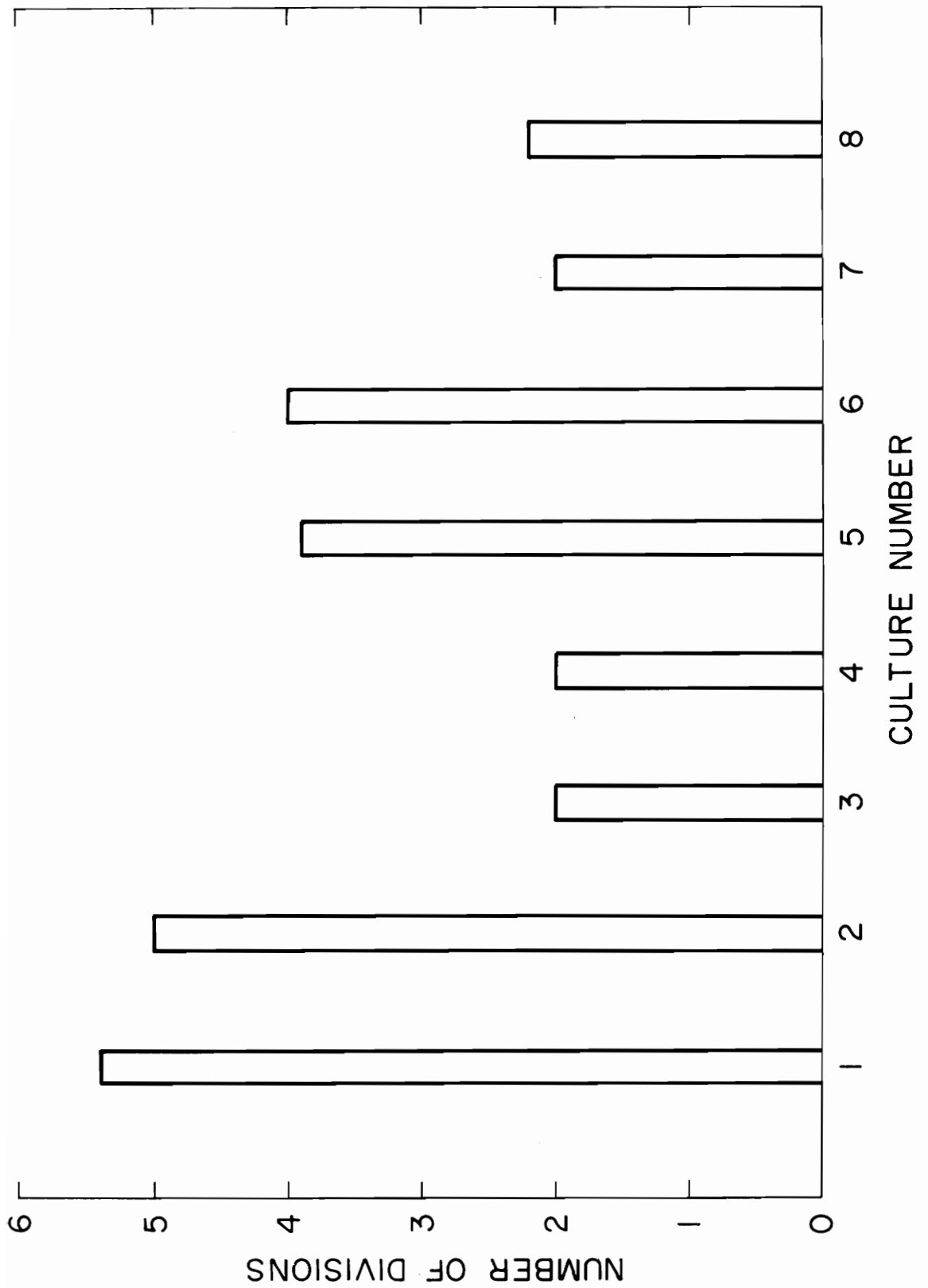
Figure 6. Summary of reproduction data for 29°C ambient populations of N. seminulum. Experiment 10. (Culture 1,2 = control, no stress; Culture 3,4 = stress 2; Culture 5,6 = stress 1; Culture 7,8 = stress 3).


NUMBER OF DIVISIONS
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CULTURE NUMBER
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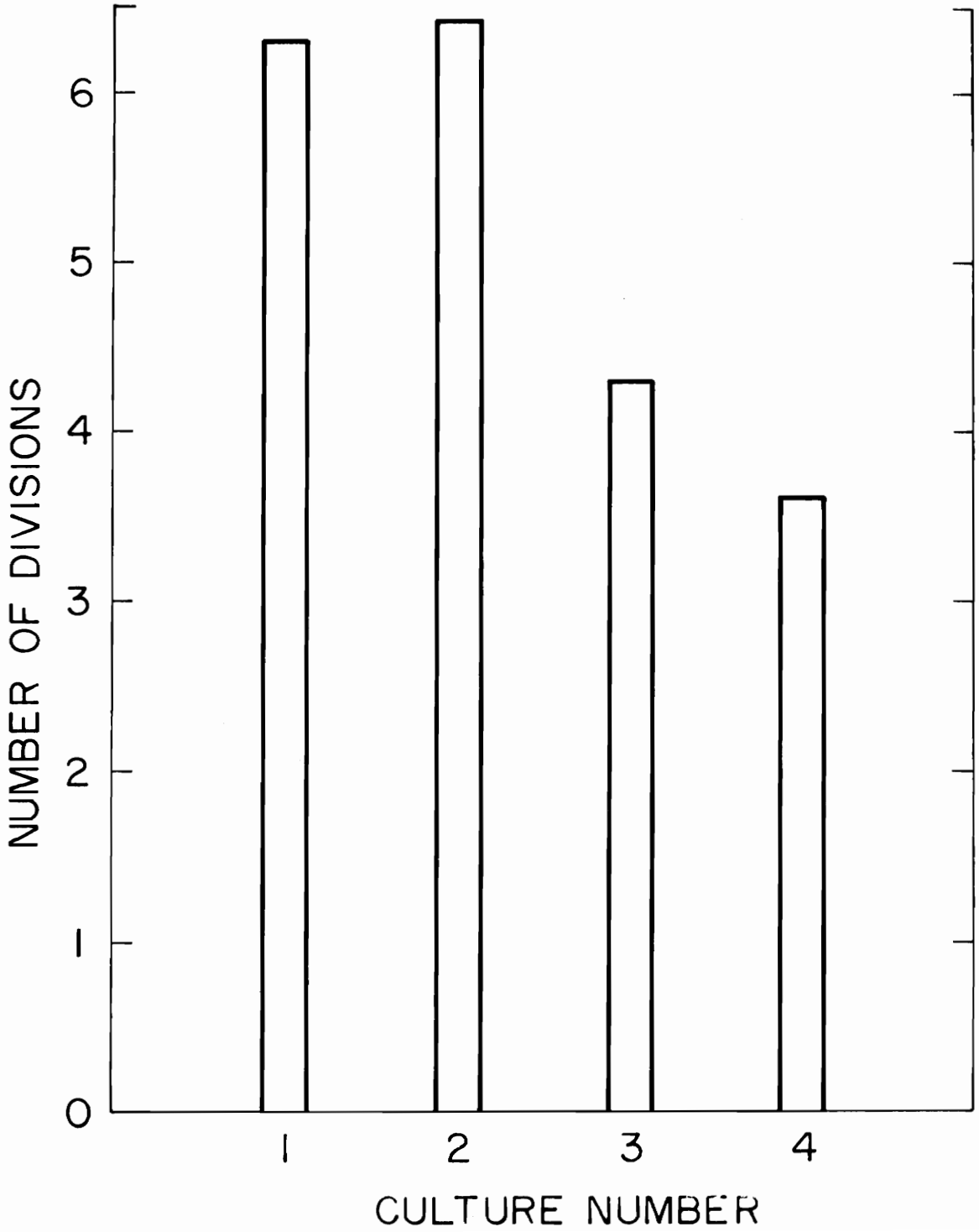
Figure 7. Summary of reproduction data for 29°C ambient populations of N. seminulum. Experiment 13. (Culture 1,2 = control, no stress; Culture 3,4 = stress 4).


NUMBER OF DIVISIONS
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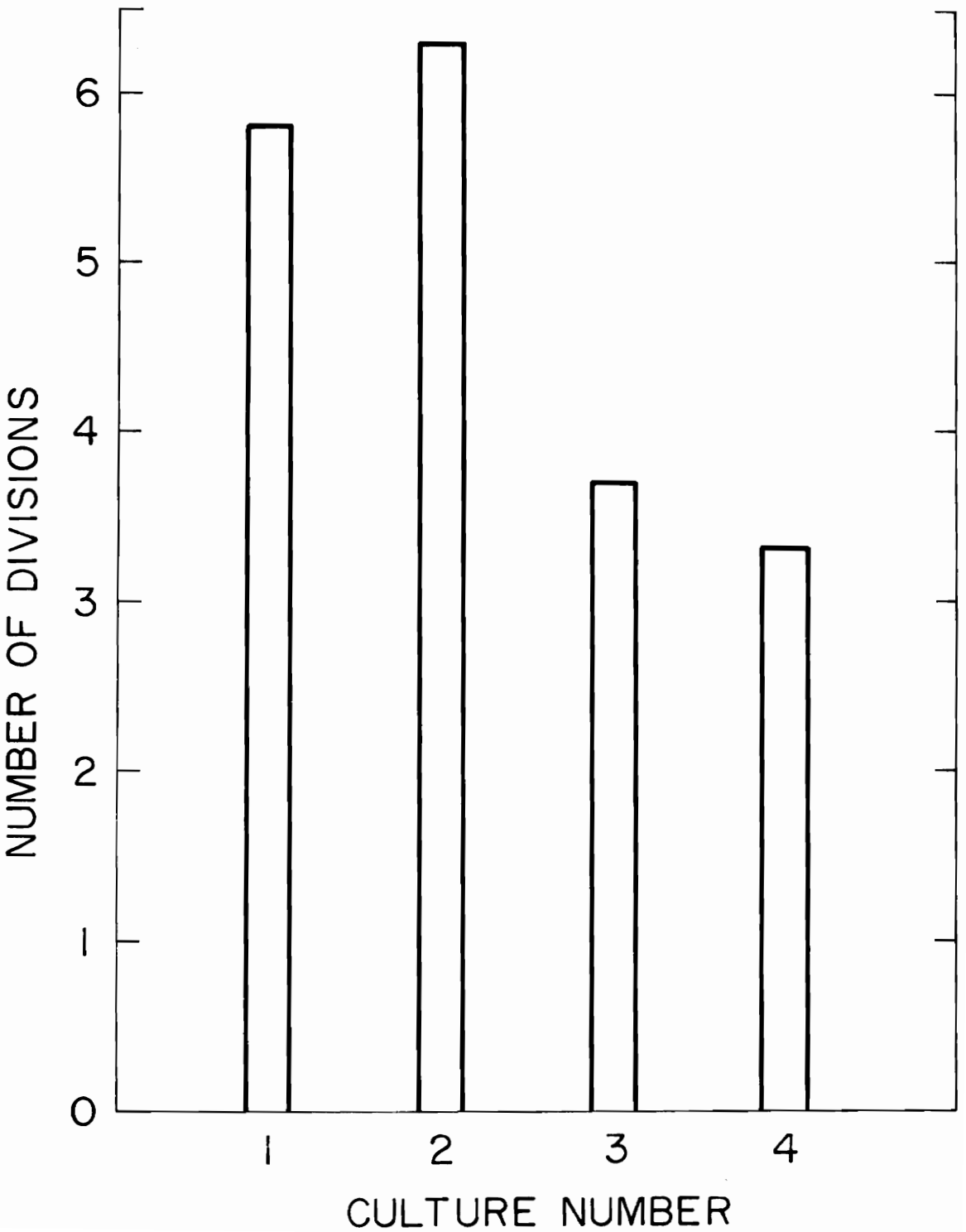
CULTURE NUMBER

Figure 8. Summary of reproduction data for 29°C ambient populations of N. seminulum. Experiment 14. (Culture 1,2 = control, no stress; Culture 3,4 = stress 4).


NUMBER OF DIVISIONS
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CULTURE NUMBER

TABLE I. DATA SUMMARY OF ULTRAVIOLET MICROSCOPIC SURVEYS OF 18°C AMBIENT POPULATIONS ON N. SEMINULUM RECEIVING STRESSES 1-3 ON DAY 3. EXPERIMENTS 1 AND 2.

Population Number	Treatment Stress 1	Treatment Stress 2	Treatment Day	Percent Altered Cells per 1000 Experiment No. 1	Experiment No. 2
1	--	--	--	3.0	1.2
2	--	--	--	1.0	3.3
3	--	+	3	2.2	2.4
4	--	+	3	1.4	1.6
5	+	--	3	3.2	2.3
6	+	--	3	1.5	1.6
7	+	+	3	2.4	2.8
8	+	+	3	3.6	0.4

TABLE II. DATA SUMMARY OF ULTRAVIOLET MICROSCOPIC SURVEYS OF 18°C AMBIENT POPULATIONS OF N. SEMINULUM RECEIVING STRESS 1 ON DAY 7. EXPERIMENTS 3 AND 4.

Population Number	Treatment Stress 1	Treatment Day	Percent Altered Cells per 1000 Experiment No. 3	4
1	--	--	1.3	3.1
2	--	--	4.2	0.2
3	+	7	0.8	2.3
4	+	7	1.8	1.0

TABLE III. DATA SUMMARY OF ULTRAVIOLET MICROSCOPIC SURVEYS OF 23°C AMBIENT POPULATIONS OF *N. SEMINULUM* RECEIVING STRESSES 1-3 ON DAY 3. EXPERIMENTS 5 AND 6.

Population Number	Treatment Stress 1	Treatment Stress 2	Treatment Day	Percent Altered Cells per 1000 Experiment No. 5	Experiment No. 6
1	--	--	--	1.3	2.5
2	--	--	--	0.0	0.5
3	--	+	3	1.5	1.7
4	--	+	3	1.2	0.3
5	+	--	3	1.3	2.0
6	+	--	3	2.2	0.5
7	+	+	3	1.0	1.2
8	+	+	3	3.2	0.3

TABLE IV. DATA SUMMARY OF ULTRAVIOLET MICROSCOPIC SURVEYS OF 23°C AMBIENT POPULATIONS OF N. SEMINULUM RECEIVING STRESS 1 ON DAY 7. EXPERIMENTS 7 AND 8.

Population Number	Treatment Stress 1	Treatment Day	Percent Altered Cells per 1000 Experiment No. 7	8
1	--	--	0.2	0.3
2	--	--	0.1	1.3
3	+	7	0.0	0.4
4	+	7	0.1	0.2

TABLE V. DATA SUMMARY OF ULTRAVIOLET MICROSCOPIC SURVEYS OF 29°C AMBIENT POPULATIONS OF *N. SEMINULUM* RECEIVING STRESSES 1-3 ON DAY 3. EXPERIMENTS 9 AND 10.

Population Number	Treatment Stress 1	Treatment Stress 2	Treatment Day	Percent Altered Cells per 1000 Experiment No. 9	Experiment No. 10
1	--	--	--	2.9	0.9
2	--	--	--	0.5	0.3
3	--	+	3	99.5	99.5
4	--	+	3	99.8	99.8
5	+	--	3	0.3	0.3
6	+	--	3	0.1	1.0
7	+	+	3	99.7	99.8
8	+	+	3	100.0	99.3

TABLE VI. DATA SUMMARY OF ULTRAVIOLET MICROSCOPIC SURVEYS OF 29°C AMBIENT POPULATIONS OF N. SEMINULUM RECEIVING STRESS 1 ON DAY 7. EXPERIMENTS T1 AND T2.

Population Number	Treatment Stress 1	Treatment Day	Percent Altered Cells per 1000 Experiment No. 11	12
1	--	--	1.5	0.3
2	--	--	2.1	0.8
3	+	7	0.8	0.1
4	+	7	1.2	0.5

TABLE VII. DATA SUMMARY OF ULTRAVIOLET MICROSCOPIC SURVEYS OF 29°C AMBIENT POPULATIONS OF N. SEMINULUM RECEIVING STRESS 4 ON DAY 3. EXPERIMENTS T3 AND T4.

Population Number	Treatment Stress 4	Treatment Day	Percent Altered Cells per 1000 Experiment No. 13	14
1	--	--	0.8	0.2
2	--	--	0.5	1.1
3	+	3	99.7	99.1
4	+	3	87.2	96.6

TABLE VIII. DOMINANT DIATOM COMMUNITY COMPONENTS COLLECTED AT 20°C IN THE NEW RIVER, GILES COUNTY, VIRGINIA, 1970. EXPERIMENTS 25 AND 26

Species
1. *Gomphonema parvulum*
2. *Nitzschia frustulum*
3. *Cocconeis placentula* var. *euglypta*
4. *Fragilaria capucina*
5. *Synedra rumpens*
6. *Navicula salinarum* var. *intermedia*

TABLE IX. DOMINANT DIATOM COMMUNITY COMPONENTS COLLECTED AT 22°C IN THE NEW RIVER, GILES COUNTY, VIRGINIA 1970. EXPERIMENTS 27 AND 28

Species
1. *Gomphonema parvulum*
2. *Cocconeis placentula* var. *euglypta*
3. *Nitzschia amphibia*
4. *Gomphonema longiceps* f. *gracilus* Hustedt.
5. *Navicula* sp. 1
6. *Navicula* sp. 2
7. *Fragilaria construens* var. *subsalina* Hustedt.
8. *Gomphonema* sp.
9. *Nitzschia frustulum*

TABLE X. DOMINANT DIATOM COMMUNITY COMPONENTS COLLECTED AT 9-10°C IN THE NEW RIVER, GILES COUNTY, VIRGINIA, 1970. EXPERIMENTS 29-34.

Species
1. Diatoma vulgare Bory. var. breve Grun.
2. Fragilaria capucina Desmazieres
3. Achnanthes lanceolata (Breb.) Grun.
4. Nitzschia thermalis (Kuetzing) Grun.
5. Navicula cryptocephala var. veneta (Kuetz.) Rabh.
6. Cymbella turgida (Gregory) Cleve.
7. Gomphonema parvulum (Kutz.)
8. Fragilaria brevistriata Grun var. brevistrista
9. Fragilaria intermedia Grunow
10. Synedra rumpens var. familiaris (Kutz.) Grun.
11. Nitzschia amphibia
12. Synedra tabulata Pant.
13. Nitzschia frustulum (Kutz.) Grun.
14. Achnanthes biasolettiana (Kutz.) Grun.
15. Cocconeis placentula var. euglypta
16. Achnanthes linearis F. curta H. L. Sm.
17. Navicula salinarum var. intermedia (Grun) Cl.
18. Cocconeis placentula var. lineata (Ehr.) V. H.
19. Achnanthes lanceolata var. rostrata (Ostrups) Hustedt.
20. Navicula tripunctata var. schizonemoides (V. H.) Patrick
21. Gomphonema helveticum Brun.

TABLE XI. DOMINANT DIATOM COMMUNITY COMPONENTS COLLECTED AT 15-16°C IN THE NEW RIVER, GILES COUNTY, VIRGINIA, 1970. EXPERIMENTS 35 AND 36.

Species
1. *Fragilaria capucina*
2. *Gomphonema parvulum*
3. *Nitzschia frustulum*
4. *Cymbella turgida*
5. *Achnanthes lanceolata*
6. *Fragilaria intermedia*
7. *Gomphonema ventricosum* Gregory
8. *Synedra tabulata* (Ag.) Kutz.
9. *Cyclotella glomerata* Bachmann
10. *Cocconeis placentula* var. *euglypta*
11. *Synedra hyperborea* var. *rostellata* Grunow and Wien

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PHYSIO-MORPHOLOGICAL EFFECTS OF ABRUPT THERMAL STRESS ON DIATOMS

by

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(ABSTRACT)

The physio-morphological effects of several categories of defined abrupt temperature increases on diatoms were evaluated. Autecological and synecological studies involving various ambient temperatures and culture conditions were examined. Populations were maintained in laboratory culture at three ambient temperatures within the optimum growth range of the species; i.e. 18, 23, and 29°C, and subjected to various categories of thermal stress. Stresses were designed to generally simulate abrupt temperature shocks which could result from entrainment through cooling lines of electric power generating facilities and downstream additions of thermal effluents. Other populations growing at 20°C were prestressed by maintenance in sub-optimal culture and exposed to added stress in the form of temperature increases. Synecological studies on diatoms from field collections under various normal and sub-optimal culture maintenance were also carried out for comparison with single species populations.

Established criteria such as light microscopic surveys on cell condition and effects on population growth (autecological studies only) were carried out. In addition, a new criterion involving total

cellular fluorescent patterns; i.e. the autofluorescence of metallo-porphyrins and the induced fluorescence of 3, 4 Benzpyrene - Caffeine fluorochromed lipid, was developed and initially tested as a new technique in measuring physio-morphological cellular alterations following thermal stress. Changes in cellular fluorescent patterns prior to, and following severe internal cellular destruction of diatoms are discussed along with certain ecological and physiological implications.