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Limnodrilus Hoffmeisteri: Observations of the Coelomic Cells and Stage I Sperm under Laboratory Conditions, Field Conditions, and After Exposure to Several Levels of Radiation

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LIMNODRILUS HOFFMEISTERI: OBSERVATIONS OF THE COELOMIC CELLS AND STAGE I SPERM UNDER LABORATORY CONDITIONS, FIELD CONDITIONS, AND AFTER EXPOSURE TO SEVERAL LEVELS OF RADIATION.

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INTRODUCTION

Annelids belonging to the family Tubificidae are distributed in the bottom substrates of streams and lakes throughout the world. These forms are particularly abundant in areas of organic enrichment. Tubificids have been shown to be important as biological-indicators of organic pollution due to the fact that they are very resistant to the industrial and domestic pollutants that are dumped into our waterways each year. They are also able to flourish in waters of very low oxygen content (Palmer, 1968).

The genus Limnodrilus Claparède is one of the most studied groups in this family. This is probably due to the great numbers of individuals of this genus that can be found in organically enriched streams near large metropolitan areas as well as smaller population centers (Goodnight and Whitley, 1960; Brinkhurst and Jamieson, 1971; Kennedy, 1966a, 1966b).

The worms used in the present study are members of the genus Limnodrilus. Taxonomically, these worms are identified by the occurrence of dorsal and ventral setae consisting of bifid crochets, spermatophores (spermatozeugma), long vas deferans, and an atrium with solid prostate. Specific criteria are found in the size and shape of the paired chitinous penis sheaths. This taxonomic character requires sexual maturity of the worm. Variation does occur in the penis sheaths, and this can cause identity confusion. Limnodrilus hoffmeisteri Claparède shows the least variation and is the species used in this study.

Numerous investigations have been undertaken on worms of the genus Limnodrilus and Tubifex. Both are members of the sub-family Tubificinae. Studies include ecological distribution in areas subjected to heavy sewage outfalls (Gaufin and Tarzwell, 1956; Hiltunen, 1969; Appleby and Brinkhurst, 1970); resistance of the worms to various industrial effluents (Ito and Kuwada, 1964; Whitten and Goodnight, 1966a; Whitley, 1968); worm effects due to the availability of oxygen within a stream ecosystem (Por and Masry, 1968; Walker, 1970); uptake and retention of various isotopes by the worms (Tomiyama, Ishio and Kobayashi, 1956; Whitten and Goodnight, 1967, 1968); the value of these worms as nutritive source in relation to fish productivity (Saddler, Drueger, Tinsley and Lowry, 1967; Kennedy, 1969); viability of the organism in the presence of antibiotics (Rice, 1939; Color, Gunner and Zuckma, 1968); and the effects of X-irradiation on the regeneration processes of worm excised tissues (Stone, 1932, 1933). Other studies on Tubificinae have included their life histories (Kennedy, 1966b; Dixon, 1915), anatomical structure at the light microscopical level (Gatenby, 1916), and a general examination of their coelomic cells (Dixon, 1915; Stephenson, 1930; Cook, 1969).

The coelomic cells have interested researchers for a number of years. This interest may be due to some unique properties of these cells. For example, certain of these cells in some of the invertebrates are believed to be capable of trephocytic actions (nurse cell) and then be phagocytic or important in wound healing (Stone, 1932; Liebman, 1947; Roots, 1957, 1960; Andrews, 1965). Such cells are thought to be capable of differentiating to meet a particular need

during stress. Little information is available on the coelomic cells of Limnodrilus. The responses of the worm to various stresses, including pollutants, have been studied; but the action of the coelomic cells during these stress periods has not been investigated.

A few studies have morphologically identified the coelomic cells of several species of the sub-family Tubificinae. These investigations were for the most part obtained from chance observations through the characterization of new species. Information on the coelomic cells in regard to numbers, ratios of one cell to another or changes in ratios of these cells during environmental stress or under laboratory conditions have not been compiled (See Stephenson, 1930).

These worms are known to exist in great abundance in the bottom sediments near some nuclear reactors. In all probability these worms have been subjected to great amounts of ionizing radiation (small doses over long periods of time). It is known that these organisms survive high doses of such radiation (Stone, 1932, 1933). However, studies have not quantitated such effects on the coelomic cells or recorded any observable morphological effects on the cells under radiation stress. Spermatogenesis and oogenesis has not been observed under radiation stress.

The importance of these worms as indicator organisms suggests that studies are needed at the cellular level in order to better understand the physiological processes that enable these worms to withstand the stresses of radiation and exposure to many industrial effluents. The present study is designed to determine: 1. A morphological identification of the coelomic cells of Limnodrilus

hoffmeisteri, 2. a quantitative analysis of the different cells under seasonal change as observed in their habitat, and 3. the effects of X-irradiation on the coelomic cells and on primary spermatocytes as observed under laboratory conditions.

LITERATURE REVIEW

The Biology of Limnodrilus hoffmeisteri and related Tubificinae

Kennedy (1966b) described the life history of Limnodrilus hoffmeisteri. Three groupings were used to characterize the age of the worm: 1. immature - no penis sheath, 2. mature - penis sheath present, and 3. breeding - spermatophores (spermatozeugma) present. A fourth grouping includes the presence of coccoons on the worms. Local conditions are said to cause variation in the breeding season. However, an annual peak was observed by Kennedy in late winter and early spring for worms inhabiting selected streams in England. Kennedy's studies suggested that the worm matured in from six months to two years and would breed in the first and second year of life. Most worms died after breeding while others were capable of returning to the immature stage and subsequently mature again and breed, often, in the same year or a following year. His studies showed that age determination was very difficult if not entirely impossible.

The systematics for this species have been thoroughly studied (Brinkhurst and Jamieson, 1971). Limnodrilus hoffmeisteri is placed in the sub-family Tubificinae along with seven other genera: Tubifex, Isochaeta, Peloscolex, Potomothrix, Psammoryctides, Antipodrilus, Ilydorilus. The sub-family is characterized by atria with an attached single solid prostate and the presence of spermatophores (spermatozuegma) in the spermathecae. Again, the use of this system of classification requires that the worm be in its breeding stage for positive

identification. The presence or absence of coelomocytes is used to characterize members of the sub-families of Tubificidae. The sub-family Tubificinae is reported to have no coelomocytes. This characteristic is open to question and will be considered fully in the discussion.

An extensive literature search has found only three papers dealing with X-irradiation effects on tubificid worms. Stone (1932, 1933) reported on the effects of X-rays on the regeneration of excised anterior and posterior segments for Tubifex tubifex (Muller). Worms with excised posterior segments were placed under an X-ray tube that delivered 237.5 R/minute for varied time intervals. Twenty-five minute exposure reduced the number of worms capable of regenerating posterior segments to a very few, and a thirty minute exposure was reported to inhibit posterior regeneration. The worms after a thirty minute exposure were capable of wound healing with a formation of what Stone calls an "anal knob". The regenerated segments from exposed Tubifex could not be distinguished from freshly collected individuals. Chloragogen cells appeared approximately 30 days after the beginning of regeneration. Stone suggested that these cells might have migrated into these regions.

Stone (1933) found that worms with excised anterior segments subjected to 9,000 R would not regenerate new segments. Wound healing would occur, and the worms would survive upwards of two months after exposure before death. He concluded from the X-ray studies that wound healing and regeneration of segments are independent of each other.

Dobrowolsky (1967) reported the effects of X-rays on the development of Tubifex tubifex embryos. He studied eleven stages in the embryogenesis of the worm. At the selected stage, the developing worms were exposed to 2,310 rads (r) or 4,620 r. He found normal embryogenesis to occur in the laboratory within 21 to 26 days. The process appeared to be temperature dependent. Irradiated embryos did not survive.

The chloragogen cells have been observed in oligochaetes for many years (Claparède, 1861). They were found lining the gut lumen throughout most of these worms. The often pear-shaped, highly granulated cells (Figure 2, p. 83) have been analysed for chemical composition (Roots, 1957, 1960). She found the chloragogen granules of earthworms to contain approximately 43% carbon, 6% hydrogen, 4% nitrogen, 3.5% phosphorus, and 1% sulphur. She concluded that the lack of large amounts of nitrogen in these cells indicated that these cells have little if any relationship to nitrogenous waste excretion. The results were supported by a study by Semal-Van Gansen (1956). Roots later reported phospholipids and other lipid-soluble compounds to occur in chloragogen tissue of several terrestrial earthworms.

Whitten and Goodnight (1966b) found the chloragogen tissue of Limnodrilus hoffmeisteri to take up Sudan Black B very strongly which indicated a high lipid content in these cells. Other tissues were stained lightly or not at all. Audioradiographs were examined after Limnodrilus hoffmeisteri had been exposed to radioactive phosphorous. The radiographs showed phosphorous was readily taken up in the chloragogen tissue. This information agreed with Roots (1960), and it was

concluded that the chloragogen tissue of Limnodrilus hoffmeisteri probably contains phospholipids and other lipid-soluble compounds. In addition, Djaczenko, et al (1969) utilizing the electron microscope, examined the ribonucleoprotein components in the nucleoli of chloragogen cells in Tubifex tubifex.

Roots (1957) conclusion that chloragogen cells were not involved in nitrogenous waste excretion contributes to a standing argument on the function of chloragogen cells. The argument appears to be limited to two basic line of thought: phagocytic functions and/or trephocytic functions. Three schools of thought have developed: 1. Those who believe that the chloragogen are involved in excretion and absorbing foreign or decaying material (Phagocytic); 2. Those who believe that the chloragogen are involved in nutritive functions (Trephocytic); and 3. A combination of both. These points are reviewed by Roots (1960).

Liebman (1947) described a trephocyte as a daughter cell of chloragogen tissue. These cells were reported in Tubifex and Eisenia to be a source of nutrition. Growth correlations were made after amputation of posterior segments. The trephocytes were found to migrate to the wound site and to aid in regeneration. This would deplete the number of these nutritive cells from around the eggs. Consequently, egg production would cease until the main regenerative process was over.

Other coelomic cells in members of the sub-family Tubificinae have been reported. Dixon (1915) described a clear colorless fluid with a number of colorless corpuscles for Tubifex tubifex. These corpuscles would assume ameboid or spherical shapes. She noted that

the number of these corpuscles would vary enormously among individuals. Dixon stated that chloragogen cells lined the visceral layer of the gut lumen. Stephenson (1930) briefly reviewed the literature concerning coelomic cells in Tubificidae. The ameboid cells of Tubifex were described to be lobed and approximately 20μ in diameter. One or two nucleoli were found in the nucleus. Little movement of these cells within the coelom was noted. Peloscolex benedeni was described to have coelomic cells that resembled chloragogen cells. It was noted that the chloragogen were more deeply colored than the coelomic cells. Recently, Vostal (1971) discussed the occurrence of ameboid cells in Tubifex tubifex.

Stang-Voss (1971) described two types of coelomic cells in the polychaete, Eisenia foetida. They are: 1. eleocyte and 2. amebocyte. Eleocytes are released from the chloragogen tissue. The function of this cell was nutritive or trephocytic. In early stages, the eleocyte was seen to contain chloragosomes (Figure 4, p. 85) which were later replaced by protein vacuoles containing hemoglobin and ferritin. These products were released into the hemolymph. Amebocytes were formed from the peritoneal endothelium. The cells function was described to be for transport and phagocytosis.

Two general methods for obtaining coelomic cells have been described for oligochaetes. These methods have been predominately used for terrestrial forms probably because they have been the most frequently worked with. Keng (1895) described what this paper calls reflex bleeding as a natural oozing of substances from the pores, mouth and anal areas of Lumbricus. Reflex bleeding is a normal probably

defensive response from the worm when placed in or on a foreign substance. One is familiar with the sticky mucous-like substance that clings to the hand after handling one or several of the earthworms. Enchytraeus albidus will exude coelomic fluid from the mouth and/or anal area, soon after one of these worms is placed on a glass slide, making the study of cells within these fluids very easy.

Effects of radiation on selected invertebrates

Radiation studies within the invertebrates have been primarily concerned with population and community responses, individual and species responses, and radionuclide cycling (Nelson and Evans, 1969; O'Brien and Wolfe, 1964; Grosch, 1965; Whitson, 1972). Those papers pertinent to this investigation are reviewed.

Jayaraman and Ducoff (1970) observed partial-body X-irradiation on larvae of Tenebrio molitor. They reported critical sites for lethality and delay in pupation to occur in the anterior third of the larvae. Torossian (1971) studied queenless worker ants, Dolichoderus quadripunctatus, after X- and gamma ray exposure of from 1,000 r to 150,000 r. His findings suggested that the adult is resistant to the radiation. However, ovarian tissue was very sensitive, and no matter what the dosage sterility would occur within a few days after exposure. Eggs produced by irradiated workers never survived. Cavalloro and Deiro (1971) reported no effects on the collembola, Hypogastrusa meridionales, after 10 days from gamma exposure of less than 100,000 r. Low dosages of up to 20,000 r increased the longevity of radiated organisms as compared to controls. Individual mortality was attributed

to irreparable damage to the central nervous system. Calderon and Gonen (1971) found adult Ephestia cautella longevity shortened in the male at 45,000 r. Sterility occurred for both male and female at the 99+% after exposure to 40,000 r and 45,000 r respectively. Singh (1971) studied the effects of low level gamma radiation (500 R; 1,000 R; 2,000 R) on the beetle, Rhyzopertha dominica. Oviposition was delayed, and oviposition was terminated earlier. Abnormal eggs increased, and larva from normal eggs died in 10 to 15 days.

Cooley and Miller (1971) examined chronic irradiation on populations of the aquatic, hermaphroditic snail Physa heterostropha. Small populations were placed under a continuous source of gamma radiation throughout a life span. The levels included 25 r/hour, 10 r/hour, and 1 r/hour. The 25 r/hour dose rate eliminated reproduction, and death occurred in one generation. Reproduction reduced significantly at the 10 r/hour, and the authors suggested population death after two generations. The dose rate of 1 r/hour gave indication of reduced fecundity. An examination of the ovotestis at 10 r/hour or 32,000 r total dose for the experiment showed partial atrophy. The 25 r/hour or 80,000 r total dose for the experiment showed total atrophy of the ovotestis.

Radiation and Tubificidae

Tubificid worms also known as sludge worms inhabit the bottom sediments of streams, rivers and lakes. Whitten (1966) stated that these organisms can make up to 80% of the biomass in areas receiving organic enrichment. Normally, such enriched areas of organic

pollution are located in and around human populations including residential sites, business areas, and notably industrial complexes. Whitten also noted that enriched bottom sediments may be found to contain a large proportion of radioactivity in an aquatic ecosystem. Ophel (1962) reported radioactive strontium to be contained in the top one inch of the benthic ooze in a Canadian lake. This amounted to 90% of the total radioactivity in the lake. The radioactive substance had seeped into the lake from a nearby liquid disposal area. In a similar situation, tubificid worms which inhabit these type of sediments in great numbers could receive large dosages of radiation. The effects of such radiation on Tubificids and more importantly the food web are unknown.

The International Joint Commission on the pollution of Lake Erie, Lake Ontario, and the international section of the St. Lawrence River (Campbell, 1969a, 1969b, 1969c; Heeney and Herter, 1970) have cited five main sources of radioactive substances that are dumped into the above waterways. They are: nuclear reactors, waste processing plants, industrial, medical, and research. The radioactive wastes are discharged into the lakes directly or through municipal sewers.

Further analyses of tubificid distributions in these waterways (Brinkhurst, et al, 1968; Hiltunen, 1969; Brinkhurst, 1969) shows close relationships to municipalities, nuclear reactors, and industrial sites as noted by the International Joint Commission. It would seem from the evidence cited that these sludge worms are likely to be subjected to radioactive substances that are already being placed into waterways and would potentially be subjected to accidental spills or

seepage problems. Little work has been done concerning the effects of radiation stress on these organisms.

METHODS AND MATERIALS

The methods and materials has been divided into four sections. These sections represent the different experiments conducted. The four sections are: 1. Laboratory Culturing and Surgical Techniques, 2. Methodology for Irradiation Procedures, 3. Histological and Photomicrographic Techniques, and 4. Statistical Methods.

Laboratory Culturing and Surgical Techniques

A comparison of cells from both laboratory cultures and from field samples was done. The worms maintained in the laboratory were obtained from the Portage Creek on or near the property of the Allied Paper Company in Kalamazoo, Michigan. Samples of the benthos were taken using an Ekman dredge. The worms with substrate were transferred immediately to the laboratory, placed in a well aerated five gallon aquaria, kept at a constant temperature (24° C ±1) with well water. The organisms were allowed to adapt for a period of two weeks before coelomic fluid analysis was done.

During the field studies, worms were secured as described above. Worms were taken immediately to the laboratory, separated from the sediments, and their coelomic fluids collected.

The separation of the worms from the sediments can be difficult, especially from the tube surrounding the worms. This tube is composed from surrounding sediments and from worm muco-secretions. This separation problem was solved by taking an amount of sediments containing the worms and placing both into a finger bowl. Water was not added,

and the bottom substrate was separated from the worm using forceps and dissecting needles. This exposed clumps or individual worms which were then placed into another bowl containing distilled or well water. At times tubes would still adhere to the worm, however, time and a little patience usually found that most worms would disassociate with the tube. Worm identification followed Brinkhurst's (1960) procedure. The worm was now ready for "bleeding". This procedure was undertaken so as to produce as little stress as possible that may interfere with the development and identification of coelomic cells.

The "bleeding" method involved taking a sharp instrument, piercing the cuticle through to the coelom, withdrawing the instrument and collecting the fluids as it flowed through the wound. Fluids are generally collected on glass slides for microscopic examination.

Curiously enough, the puncture technique was hindered by the worm's production of a secreted envelope. Coelomic fluid could be seen to flow from the wound but this envelope soon prevented its release onto a glass slide. A modification of the technique for this group of worms consisted of tearing the secreted envelope, and then, gently pulling the worms along the glass slide surface after a puncture. The coelomic fluids were released and air fixed.

In this study, punctures were made between segments 5 and 25 for the field and radiation studies. Segments 10 and 14, including the clitellum, were normally a little wider than the rest of the worm and were the most recognizable area from which to collect fluids. However, as the worms become reproductively mature, egg sacs and sperm sacs developed into the coelom. Thus, punctures within these segments

sometime resulted in the rupture of egg sacs and spermeries, causing their release onto the slide with coelomic fluid, thereby allowing evaluation of reproductive maturity.

Methodology for Irradiation Procedures

Worms used in this experiment were collected from Portage Creek by the Allied Paper Company in Kalamazoo, Michigan, on April 2, 1972. The water temperature was approximately 20° C, and the air temperature was around 15° C. The stream receives heated waters used by the paper company for cooling purposes. The organisms suspended in substrate were transported to the laboratory and systematically seived to a standard sieve #30 (590㎛). The worms plus the remaining substrate were placed into 10 inch diameter circular glass aquaria. Five cultures were established: Three for radiation levels and two for controls. The cultures were then placed into a Sherer environmental chamber. The Light/Dark (L/D) cycle was set at nine (9) hours light and fifteen (15) hours dark. The temperature was maintained at 20.5° C$^{\pm}$ .5° throughout the experiment. Both incandescent and fluorescent lights were used. The evenly distributed light was measured to have an intensity of 200 foot candles or lumens per square foot. A Weston Illuminator Meter Model #756 utilizing a filter to color correct for human eyes was used to measure light intensity. The filter allows accurate measurement of fluorescent and tungston light sources.

The substrate for culturing the worms consisted of a mixture of paper fibers, mud silts, decomposing vegetation, unidentified seeds, and small gravel. The substrate covered the bottom of the aquaria to

a depth of approximately 1/2 inch. A .01% Knop's solution (Table 1, p. 44) was added to a depth of 1 inch above the substrate (Whitten, 1966). The Knop's solution was changed daily to prevent bacterial contamination. The Knop's solution was stored in five gallon containers and was constantly aerated to insure available oxygen in the water for the cultures throughout the experiment. Periodic monitoring of the oxygen (mg/liter) was taken using the Hach kit modified Azide-Winkler method. Oxygen levels were normally between 4 and 6 mg/liter after a 24 hour period and never below 2 mg/liter. The cultures were allowed to acclimate in the environmental chamber four days prior to radiation exposure.

The worms were prepared for X-irradiation in the following procedure. Four (4) of the five (5) cultures were emptied of the Knop's solution. Individual cultures, including substrate and worms, were placed into Nasco "Whirly Pak" plastic containers measuring two (2) inches in width and seven (7) inches in length. The bags when filled and closed measured approximately one (1) inch in depth. This procedure was used for the placement of the sample under the radiation source. Two controls were used in this experiment. Control 1 was left in the environmental chamber untouched during the period of time needed for irradiation. Control 2 went through the same procedures as the samples for irradiation with the exception of radiation treatment. The four "Whirly Paks" were placed into a cardboard container and were then taken by automobile to the Upjohn Company in downtown Kalamazoo. The company's Van de Graaf accelerator was used to generate a source of X-rays. An individual "Whirly Pak" was placed on a conveyer belt

and mechanically moved into the target site area. A Roentgen (R) unit countdown dosage meter was strapped alongside the "Whirly Pak" to record dosage level. Individual "Whirly Paks" were "rocked" back and forth under the target site to help insure uniform dosage throughout the sample. The "rocking" procedure involved changing the direction of the conveyer belt, a mechanism built into the instrument.

Three dosage levels were administered. The levels were: 1. 7,000 R, 2. 3,000 R, and 3. 1,000 R. Initially, each "Whirly Pak" received 10 per cent of its total dosage. This procedure was used due to a miscalculation of R units administered. Individual "Whirly Paks" were again placed in the target site and subjected to the remaining 90 per cent of the dosage level. Approximately one hour elapsed from the time the first and last "Whirly Pak" was irradiated. Time zero was established at 10:00 AM eastern standard time, April 6, 1972.

The irradiated series and control worms were taken back to the laboratory, placed into individual glass aquaria, Knop's solution added, and culture maintenance followed the procedure described above (Figure 1, p. 82).

Ten (10) Limnodrilus hoffmeisteri from each aquaria were taken from the cultures at prescribed time intervals (Table 12, p. 58). The coelomic fluid was placed on glass slides. Worms were identified primarily on the basis of the absence of hair or pectinate setae and the presence of bifid setae. Previous investigations in our laboratory supported this method since precise identification usually requires sacrificing the organism. On occasion the penis sheath could be

observed under the dissecting scope and positively identified as Limnodrilus hoffmeisteri. In certain cases the sample number of ten was not used because sample number could not be obtained within a predetermined 2 hour sampling, identification, and coelomic fluid collection period or when the coelomic fluids were lost in process of staining. These worms, characteristically, aggregate in water when substrate is not present, thereby, simplifying the sampling process. When worm aggregates were present, they were selected rather than isolated worms in the substrate.

Differential cell counts were made using the first 100 cells randomly observed. This method is similar to those used in vertebrate blood counts (Davidsohn and Henry, 1969). Three cell types were counted. A description of the cells can be found in the Results section of this paper.

Histological and Photomicrographic Techniques

The coelomic fluids were fixed by air drying. The fluids were stained with Wright's (Table 1), and counterstained with Giemsa (Table 2, p. 45) when photographs were desired. The following time schedule was used for this study:

Wright's Stain	6 - 8 minutes
Wright's Buffer	12 - 16 minutes
Giemsa	45 minutes

This procedure produced the results desired for general morphological characterization of coelomic cells using light microscopy and produced excellent contrast for photographic purposes.

Photomicrographs were taken with a Zeiss Model 1487 photomicroscope using a 35 mm film cartridge. Color photomicrographs were taken on Kodak's Kodachrome II Professional Film, Type A (KPA 135-36). This film is especially designed to be used with photoflood lamps and yields excellent resolution. Processing and development was done commercially.

Panatomic-X, a fine grain panchromatic film, was used for black and white photomicrographs. This film is especially good for enlarging without loss of image sharpness. Prints were made using Kodabromide F2 and/or Kodabromide F4 paper. The developer for the paper was Dektol (1:2 dilution), and for the film was Microdol-X (full strength); both used according to conventional film specifications.

Statistical Methods

All statistical methods were completed using the PDP-10 time sharing computer located on the campus of Western Michigan University. Consultation with the computer center's statistician provided the test procedures used in the analytical work.

An analysis of variance (ANOVA) was used for seasonal field data and laboratory data. Individual cell groups were compared with each other for the given time period (Tables 5, 6, 7, 9, 10 and 11, pp. 49-57). Barlett's test was used for analysing homogeneity of variance.

A two way analysis of variance was used to analyse the data from the radiation experiment. Time and dosage levels were the two factors analysed. The individual radiated cell groups were compared with each other. Bartlett's test for homogeneity was used to test the data.

The chi square values obtained suggested high variance of this data between individual organisms and their respective cell groups. This would have suggested that the analysis would have to be viewed cautiously. However, Winer (1971) pointed out that homogeneity of error variance can be satisfied through a proper transformation of the data provided certain criteria are met. In this case there was a high variance of a binomial distribution of individual worm cell groups. (This normally occurs in nature.) Since the binomial distribution in this case was proportional, the sample size was made into a single observation. Based on this proportional binomial an arcsin transformation was completed for stabilizing variance. The procedure follows:

$$X'_{ijk} = 2 \arcsin \sqrt{X_{ijk}}$$

where X_{ijk} is a proportion and X'_{ijk} is an angle measured in radians. Although, there is no precedent for this type of analysis in this field of radiation biology, this procedure was used for final analytical work in order to determine if there were changes in cell numbers due to the level of radiation administrated or if there were changes in cell numbers over time after irradiation.

After the two way analysis was completed, a two sample T's test for variance was used to determine where a significant change occurred. For instance, a time series was analysed to show at the various levels of radiation where changes in cell numbers had occurred. This information was summarized with the formation of a line separation table (Winer, 1971) that provides graphic representation along the time series where changes in cell numbers had occurred (See Line Separation

Technique Explanation preceding Table 19, p. 74).

The level of significance for all analytical work was at the .05 level.

RESULTS

The results have been divided into four sections. These sections represent the different experiments conducted. The four sections are: 1. Stage I sperm and coelomic cells of Limnodrilus hoffmeisteri, 2. Laboratory cultures of worms - Differential cell counts (sperm and coelomic cells) over an extended period of time, 3. Field conditions - Differential cell counts (sperm and coelomic cells) over the season of the year, and 4. Radiation experiment - Effect of X-irradiation on Stage I sperm and coelomic cells and on differential counts.

Stage I sperm and coelomic cells of Limnodrilus hoffmeisteri

The coelomic cells of Limnodrilus hoffmeisteri can be grouped into two categories: 1. The amebocyte or lymphocyte, and 2. the chloragogen. The term, amebocyte, will be morphological in use and will not imply a functional or physiological role. Amebocyte as used in this study will be a collective term for those cells that arise from one or more tissues other than chloragogen and that migrate within the coelom. The lymphocyte and the amebocyte sometimes are synonymous terms (Vostal, 1971). The amebocytes observed in Limnodrilus hoffmeisteri were found to be consistently of one form (Figures 3 and 4, pp. 84 and 85). The rounded cells were approximately 20 μ in diameter and contained a distinct acidophilic nucleus. A nucleolus sometimes was visible using light microscopy. The cytoplasm stained with basic dyes. The amebocytes were few in number and varied from one individual to another (Tables 4 and 8, pp. 47 and 51).

Chloragogen cells arise from the chloragogenous tissue which is a modification of the visceral peritoneum. These cells are easily identified by their dark granules known as chloragosomes (Figures 2, 3 and 4). The chloragosome granules are similar in size throughout the chloragogen cells. The chloragogen cell is quite large, 40 μ in diameter, with a distinct nucleus which is acidophilic. The cytoplasm is basophilic. These cells can be found throughout the coelom and have been observed within the developing egg sac.

The morphological stages of spermatogenesis have not been described in Limnodrilus hoffmeisteri. Although, a description of spermatogenesis was an original goal of this investigation, the problem became very complex and too time consuming to complete. Therefore, this study identified a Stage I sperm which has been reported to be associated with primary spermatocytes (Dixon, 1915). This type of sperm was the only consistent stage of development observed. Stage I sperm occurred as an aggregation of approximately 25 cells which associated into a morula-like structure (Figures 3 and 4). The nucleus of each cell was distinct and took an acid stain medium. The cytoplasm stained in a basic medium.

Laboratory cultures of worms - Differential cell counts (sperm and coelomic cells) over an extended period of time

An analysis of variance design was setup to measure two variables: 1. Was there significant differences in cell numbers over time? 2. Did the worms vary during the time period of the experiment?

The experiment began in early winter and was terminated in

midsummer. Table 4 shows the raw data for numbers of cells and the dates used for collection of coelomic fluids. Tables 5 and 6 indicate significant differences in cell numbers for both the chloragogen cells and the amebocytes over time. In table 7 it can be seen that there were no significant changes in numbers of morula structures observed for Stage I sperm. As can be seen from tables 5 and 6, worm variation was not significant enough to reject the F statistic at greater than the .05 level of significance. In table 7 worm variation was significant so that the F statistic at greater than the .05 level of significance was rejected.

Cell sizes and shapes did not change during this study over time.

Field conditions - Differential cell counts (sperm and coelomic cells) over the seasons of the year

The sample size of Limnodrilus hoffmeisteri used for the cell studies in this experiment was taken from a group of 100 randomly collected worms. Table 3, p. 46, shows the percentage of Limnodrilus hoffmeisteri among the Tubificidae found in the benthos of Portage Creek.

The same test and questions as stated above were used on the data collected from the field studies. The raw data for numbers of cells and the dates used for collection of coelomic fluids is shown in Table 8. Tables 9, 10 and 11 are ANOVA tests to measure significant differences in cell numbers for the chloragogen, amebocytes, and Stage I sperm from early fall through summer. Worm variation (Tables 9, 10 and 11) was not significant enough to reject the F statistic at greater than the .05 level of significance.

The numbers of individual cells from chloragogen, amebocytes, and Stage I sperm varied from one organism to another. Since the homogeniety tests of variance was rejected, the information in Tables 9, 10 and 11 has to be viewed cautiously.

Cell sizes and shapes did not change during the time of this study.

Radiation experiment - Effect of X-irradiation on Stage I sperm and coelomic cells and on differential counts

Few behavioral changes among the worms were noted during the course of the experiment. The worms within the "Whirly Paks" appeared to remain randomly distributed while being exposed to the x ray. One exception was the 3,000 R group in which the worms had previously clumped near the top of the "Whirly Pak" (nearest the beam of x rays). After exposure to the radiation, the worms had migrated to the bottom of the "Whirly Pak" or away from the x ray source. Throughout this experiment the worms remained sensitive to light, touch, or other abnormal shock. Characteristically, the worms would withdraw into their tubes or clump together when disturbed. They respired in the normal inverted position.

Two major changes were observed in the three cell groups under study. The chloragogen cells became heavily granulated and intensely black (Figure 8, p. 89). Observations of the gut lumen containing chloragogen tissue again showed a "charcoal black" effect. The intestinal area appeared to be a "charcoal black" with intensely granulated chloragogen cells circulating in and around the parent chloragogenous tissue. The appearance of the heavy granulation in these cells occurred around time series six (170 hours post irradiation) for all

three levels of radiation. The last sample, time series nine (768 hours post irradiation), showed the "charcoal black" effect of the gut lumen due to the heavy granulation of the chloragogenous tissue. The control groups did not show this effect.

The second major change noted was the necrosis and subsequent loss of Stage I sperm. The morula cells became vacuolated and contained pyknotic nuclei (Figures 9 and 10, pp. 90 and 91). Complete deterioration of the morula structure followed. Other sperm stages were affected (Figures 10 and 11, pp. 91 and 92). The rapid deterioration and loss of this tissue formation occurred in worms exposed to each of the three levels of radiation around time series six (170 hours post irradiation) (Table 12). The number of these cells dropped dramatically after series six to almost no Stage I sperm by series nine (768 hours post irradiation) (Table 12).

Very few amebocytes were observed during this experiment (Table 12). No changes were observed in the general morphology of these cells. This observation included both radiated worms and control groups.

A two way analysis of variance, using an arcsin transformation, was set up to measure two variables against a control group: 1. Were there differences in cell numbers (compared with a control) after X-irradiation over the time series used in the experiment? 2. Were there differences in cell numbers due to the level of radiation (7,000 R, 3,000 R, 1,000 R) after exposure to a worm culture? The test was used with both control 1 and control 2. For example, a radiated cell group; ie, chloragogen; was compared with the control 1 cell group and

then with the control 2 cell group. Also, the above test was used to check for differences between the control groups.

Table 12 shows the raw data for numbers of cells and the time intervals after irradiation for this experiment. The number of amebocytes was almost negligible for both radiated and control groups (Table 12). It was not possible to test the number of amebocytes for variations due to time after radiation exposure or to analyse for differences in the level of radiation.

Time was the only variable in this experiment that showed significant differences with the chloragogen cells and for Stage I sperm when these cells were compared to both their control groups (Tables 13, 14, 15 and 16, pp. 68-71). The levels of radiation did not show significant differences (Tables 13, 14, 15 and 16). It would appear that the three dosage levels had the same effect. One exception was the chloragogen cells when compared to control group 2 (Table 15). This will receive further attention in the discussion.

Tables 17 and 18, pp. 72 and 73, comparison of control 1 against control 2, showed no significant differences in the cell groups or their respective numbers. It would appear from this information that there was little difference between control 1 and control 2 as to cell numbers in a respective cell group over the duration of this experiment.

The effects of radiation over time was shown to be positive in the ANOVA test. The question next asked was: When do the changes occur after time zero? To answer this question, a Two Sample T's Test for Variance (WMU Computer Center) was used. This test was summarized using a line separation table (Winer, 1971).

The results of this test are given in Tables 19, 20 and 21, pp. 74-77. It appears that the changes were most pronounced between time series six (170 hours post irradiation) and time series seven (338 hours post irradiation). There is a discrepancy in the 7,000 R group (Table 19), which is discussed later. The control groups showed little differences over the same time series (Tables 22 and 23, pp. 78 and 79). The amebocytes could not be analysed due to their low numbers.

DISCUSSION

The discussion of the cells of Limnodrilus hoffmeisteri is divided into the following sections: 1. Demonstration of the presence of coelomic cells in the Tubificinae, Limnodrilus hoffmeisteri. 2. Use of coelomic cells as systematic characters. 3. Demonstration of the presence of Stage I sperm in the Tubificinae, Limnodrilus hoffmeisteri. 4. A study of changes in cell morphology and numbers occurring under field conditions (seasons of the year) and under laboratory conditions. 5. A study of changes in cell morphology and numbers after X-irradiation. 6. Research questions which this work has produced.

Demonstration of the presence of coelomic cells in the Tubificinae, Limnodrilus hoffmeisteri

Occurrence of coelomic cells in Limnodrilus hoffmeisteri was demonstrated in this investigation (Figures 3 and 4). The coelomic fluids containing the coelomic cells were taken from live specimens. This procedure was useful for the identification of coelomic cells for Limnodrilus hoffmeisteri.

The coelomic fluids were collected by taking a sharp instrument, piercing the cuticle through to the coelom, withdrawing the instrument and collecting the fluids as it flows through the wound. Fluids are generally collected on glass slides for microscopic examination. This method has been used as early as Keng (1895).

The coelomocytes of Limnodrilus hoffmeisteri can be grouped into two categories: 1. the amebocyte, and 2. the chloragogen. The amebocyte (Figures 3 and 4) is a spherical cell approximately 20μ in

diameter. It contains a distinct nucleus, and a frequently visible nucleolus. The chloragogen cell is quite large, 40 μ in diameter, and contains a distinct nucleus (Figures 3 and 4). This cell is easily identified by the dark granules known as chloragosomes (Figures 3 and 4).

Use of coelomic cells as systematic characters

Some investigators have supported the existence of coelomic cells in another genus of the sub-family Tubificinae: Tubifex. Dixon (1915) described corpuscles that would assume ameboid or spherical shapes. Stephenson (1930) described ameboid cells to be variously lobed and approximately 20 μ in diameter. Peloscolex benedeni was described to have coelomic cells that resembled chloragogen cells. Recently, Vostal (1917) discussed the occurrence of ameboid cells in Tubifex tubifex.

Taxonomic identification is often times based on characters in an artificial structure. Such a key is commonly used to separate the sub-families of the Tubificidae. One of the more recent keys is that by Brinkhurst and Jamieson (1971). Brinkhurst used absence or occurrence of coelomocytes to separate certain sub-families. For instance, he states that the sub-family Rhyacodrilinae has numerous coelomocytes while the sub-family Tubificinae has no coelomocytes.

Organisms (such as the Rhyacodrilinae), which have large and abundant coelomocytes, can be easily separated using low power magnification. Other species whose characters can be identified only by sacrificing the organism - fixing and clearing of the organism with an

agent such as Ammon's Lacto Phenol (Brinkhurst, 1960) - include the sub-family Tubificinae. Brinkhurst's technique would eliminate coelomocytes from the coelom; and, hence, the absence of these cells would be reported.

Demonstration of the presence of Stage I sperm in the Tubificinae, *Limnodrilus hoffmeisteri*

A Stage I sperm was demonstrated in this investigation (Figures 3 and 4). This stage in sperm maturation for this group of organisms has been reported to be associated with primary spermatocytes (Dixon, 1915). Stage I sperm was a cohesive mass of cells numbering approximately 25 in number. This mass of cells formed a morula type of structure (Figures 3 and 4). Figure 2 shows the sperm sac which develops into the coelomic area of Limnodrilus hoffmeisteri. The sac contains the maturing sperm and can be observed between segments 10 and 14.

A study of changes in cell morphology and numbers occurring under field conditions (seasons of the year) and under laboratory conditions

The field data (Tables 8 and 11) indicates that Limnodrilus hoffmeisteri, as a population, becomes reproductively mature in early winter, since Stage I sperm occurred in large numbers only during winter months. The results (Table 8) show a rise in the numbers of Stage I sperm throughout the winter months with a peak around February. The number of Stage I sperm declined in Spring. The size and shape of Stage I sperm are illustrated in Figure 3. Although egg development was not studied in this investigation, eggs were observed

in increasing numbers of organisms during this period of time. This supports the idea that an active period of reproduction was in progress during the winter months. It is likely, that the period of reproductive activity in Limnodrilus hoffmeisteri is similar to that reported by Kennedy (1966b). He found a peak reproductive activity from winter to early spring.

Since some Stage I sperm are present throughout the year, it is possible that a low level of reproductive activity could occur throughout the year. Kennedy (1966b) reported that some individuals of the species continue to reproduce in reduced numbers throughout the year in England.

The field data for the chloragogen and the amebocytes did not show any patterns other than that there were variations in cell numbers from one organism to another, and variations among the groups of organisms from one season to another (Tables 8, 9 and 10). The general morphologic characters of these cells did not change over the seasons of the year (Figure 3).

The homogeneity tests for variance between individual worms for both the laboratory cultured worms and the worms collected in the field shows high variance of the data. When variations are significant between samples, it is not appropriate to compute a mean of those samples since one is in danger of accepting a hypothesis when it should be rejected.

Variations in a variety of characteristics among individuals have been observed for many years. Variations is reported to occur in the number of coelomic cells between individuals of the same species

(Dixon, 1915; Stephenson, 1930). Another variation is in the reproductive season, even though the population peaks in winter and early spring, individuals can be found to vary greatly from one worm to another (Kennedy, 1966a, 1966b). The statistical tests of variance used could not account for the kinds of variation observed in this investigation.

Even though variations between individuals were significant, mean numbers were computed in each of the three cell types in laboratory maintained worms. Tables 4, 5, 6 and 7 are a measurement of differential cell counts. They show significant changes in cell numbers for both the chloragogen and amebocytes during the time period studied. The Stage I sperm did not show significant changes in cell numbers.

A study of changes in cell morphology and numbers after X-irradiation

Although, at the experimental dosage of X-irradiation the adult worm survived, the sensitivity of Stage I sperm to X-irradiation was demonstrated for Limnodrilus hoffmeisteri (Figures 9, 10 and 11; Tables 14, 16 and 18). The X-irradiation resulted in sperm necrosis and deterioration. Whether these worms would be capable of breeding again and producing viable young in time is a problem that needs further study. Survival after irradiation is not uncommon in invertebrates (Torossian, 1971; Calderon and Gonen, 1971).

The chloragogen cells (Figures 5, 6, 7 and 8, pp. 86-89) showed progressively increasing granulation, such that a cell membrane and nucleus were not easily seen in time series 9 (768 hours post irradiation). Initial examination of Figures 7 and 8 would suggest that the

cells had become necrotic. However, the cell membrane and nucleus can be seen by carefully focusing through the depth of individual cells. Because of photographic problems, it is not possible to show both the intense granulation, and the intact cell in the same plane. The field was focused to show the clearest images possible for both granulation and cytoplasmic organelles.

Additional chloragogen cell numbers were noted by Stone (1932, 1933) for wound healing caused by X-irradiation. Since wound healing or cellular regeneration was not noted after X-irradiation, this author would suggest that the chloragogen cells assumed a phagocytic function. Perhaps the chloragogen cells became heavily granulated following phagocytosis of sperm packets which began to deteriorate after the X-irradiation. The cells were seen to line the alimentary canal. In this fashion the cells possibly could excrete or store this phagocytosed material. This statement needs additional investigation; but, initially, it would seem to give support to the argument that these cells can under certain unspecified physiological conditions serve either as a nurse cell or develop phagocytic actions. Leibman (1947) supports the trephocytic function of such cells during regeneration or wound healing in Tubifex. Such an idea gains support through Andrews (1965) who reviewed the unusual characteristic of totipotency in oligochaete's coelomic cells. This capability and the conditions which may initiate such a response are not yet understood.

The evidence against such phagocytic properties was concluded by Roots (1957, 1960). She concluded that the lack of large amounts of nitrogen in these cells indicated that these cells have little if any

relationship to nitrogenous waste excretion. The results were supported by a study by Semal-Van Ganses (1956).

The ameboid cells remain an enigma in this study. The almost entire lack of these cells throughout the entire experiment could not be explained. Initially, it was thought that these cells would increase in numbers after radiation, if the function of these cells is similar to vertebrate lymphocytes whose numbers do increase after radiation injury. This did not occur (Table 12). The amebocytes observed in this investigation were similar to a cell described by Stone (1932, 1933). He reported a cell, which he called a neoblast, was prominent in wound healing and regeneration in Tubifex after X-irradiation. This cell was seen to migrate along the ventral nerve cord to the injured site. Whether such a cell with such a functional role exists in Limnodrilus hoffmeisteri is not known. An interesting point is seen when comparing amebocytes in the environmental worms with the laboratory worms (Tables 4, 6, 8 and 10). The complete absence of identifiable amebocytes was noted at different times of the year as well as between individual worms during times of the year when amebocytes were generally present (Tables 4 and 8). Amebocytes were never in great numbers and varied greatly from one organism to another. If there is a relationship between this amebocyte and its possible function for a regenerative role, then one would expect the cell to appear only during periods of wound healing and the development of new segments. Otherwise, one would see low numbers and high variability of these cells between organisms.

Tables 13, 14, 15, 16, 17 and 18 shows that the levels of

radiation used in this experiment had no significant effect on the differential cell counts for chloragogen and Stage I sperm. The same tables show that there are significant changes in differential counts over a period of time. Time was the more significant factor in alteration of cell numbers. The next question asked was when in the time series does change in cell numbers occur or begin to occur? For this purpose, a Two Sample T's Test for Variance was used. Essentially, the mean of each time series was placed in ascending orders and comparisons made. A graphic representation in the form of line separations clearly shows the relationships of the cell group means along the time series (Tables 19, 20, 21, 22 and 23). The information suggests that a change in cell numbers began to occur or occurred between time series 6 (170 hours post irradiation) and time series 7 (338 hours post irradiation). However, a discrepancy exists in Table 19. The previously analysed data suggested significant associations of series 3 and 8 but suggested no significance between series 6 and 7. There are two statistical situations that will set up a condition of rejection between two means using this test procedure. If there are unequal sample sizes or there is a shift in variance size, a rejection of a means association is recorded. In this case time series 6 and 7 are rejected due to the high variance in size factor. Time series 3 and 8 are seen to be associated in this test procedure. A line can be drawn through series 6 and 7 showing a significant relationship at .05 level even though it does not prove to be true. Such statistical procedure is necessary for this analysis. If, however, the line is eliminated between series 6 and 7, a definite change in cell numbers is

still seen (Tables 20 and 21).

A line is not drawn under time series 9 in Table 20 because the test will calculate means that are greater than 0 and less than 100.

An important question that was answered using this technique suggested that Control 1 and 2 used for the radiation experiments showed little change over the time series (Tables 22 and 23). Control 1 showed the least variation. Control 2 was more variable but this was a gradual trend as the lines overlapped considerably more than the irradiated cultures of Limnodrilus hoffmeisteri. This data supports the idea that the primary cause for shifts in the numbers of cells was X-irradiation since the controls had relatively the same numbers of cells throughout the experiment. Tables 17 and 18 analysed Control 1 with Control 2 to see if there were significant differences between the control groups in regards to cell numbers. The analysis showed that there were no statistical differences.

Research questions for future investigations

1. Life history of Limnodrilus hoffmeisteri: This study has suggested that a major reproductive cycle occurs in winter and early spring for worms in the Portage Creek area. Apparently, these worms were responding to a biological rhythm. What initiates this rhythm has not been studied. One aspect to study concerning a reproductive rhythm would be the role that light may have in initiating or in repressing gonadal development in these worms.

The control groups used in the radiation experiment were placed into the environmental chamber under an early winter light/dark (L/D)

cycle. The worms had been collected from the stream under a spring L/D cycle. At the beginning of the radiation experiment, the sperm observed in the control worms consisted of all developmental stages. By the end of the radiation experiment, the sperm from the control worms was generally that of the previously described Stage I spermatocytes. Did the change of the L/D cycle from spring to early winter initiate a regression of spermatogenesis to the Stage I sperm which is one of the first observable stages in sperm development in these worms? Commonly, Stage I sperm is the major cell type observed in early winter as compared to other more advanced stages is sperm development. The other stages become more prominent in mid and late winter.

To test this idea of a light role in gonadal development, a Two Sample T's test was completed using the early winter (December) field differential cell counts and the control groups used for the radiation experiment differential cell counts for Stage I sperm. This information was summarized using the Line Separation Technique (Winer, 1971). Tables 24 and 25, pp. 80 and 81, show that the mean numbers of Stage I sperm from the early time series of the radiation experiment are different than the time series used towards the end of the experiment. The early winter Stage I sperm cell counts (represented by the number 10) appear to be related to both groups. There is a trend established towards a reduction in the number of Stage I sperm in the controls and this may be caused by the L/D early winter cycle used for the radiation experiment. The trend towards reduced numbers in Stage I sperm is not clear cut and, certainly, the true relationship between the control groups used in the radiation study and the early winter Stage I sperm

cell counts cannot be fully established.

However, it would appear: 1. that with the number of stages in sperm development having been reduced to primarily the Stage I sperm by time series 9 for the control groups used in the radiation study, and 2. that with the trend that shows two groups of time series that are related by mean Stage I sperm numbers and are simultaneously related to the early winter (December 12, 1970) seasonal mean numbers of Stage I sperm, a L/D cycle may play a significant role in gonadal development within a population of Tubificid worms.

This kind of response to a L/D cycle by these worms needs investigating before any conclusions can be established.

2. Sterility after irradiation: Populations of Limnodrilus hoffmeisteri would become extinct if radiation dosage caused sterility. However, the physiological processes of this group of worms is not well understood, and it is as yet too early to state that sterility occurred in the worms subjected to X-irradiation.

It has been reported for several invertebrates that sterility often occurs while the adult survives massive amounts of irradiation. Torossian (1971) found queenless worker ants to survive massive dosages (up to 150,000 r) of radiation while the ovarian tissue was destroyed within a few days after irradiation. Consequently, the ants could not produce a new generation, and subsequently, the population died by sterility from irradiation. Calderon and Gonen (1971) and Singh (1971) reported similar findings for moths and beetles that were exposed to various levels and types of radiation. In addition, Cooley and Miller (1971) were able to show that population death would occur

in the hermaphroditic smail, Physa heterostropha, by sterilization of the adult.

Again, the fact that the Stage I sperm was destroyed by X-irradiation for Limnodrilus hoffmeisteri was demonstrated. The next question is: What effect does this have on the survival of the population of these worms? The literature supports the idea that population death would occur due to sterilization of the organism. However, these statements were made after the investigations included the complete life history of the organism that was exposed to radiation. The present study was unable to complete a life cycle of Limnodrilus hoffmeisteri since an individual worm is capable of living upwards of three years (Kennedy, 1966b). Consequently, the effect of X-irradiation on the survival of Limnodrilus hoffmeisteri as a population requires further investigation.

CONCLUSIONS

1. Coelomic cells were demonstrated for Limnodrilus hoffmeisteri. Two cell types were described: 1. Amebocyte, and 2. Chloragogen. The chloragogen cell is formed from parent chloragogenous tissue which is a modification of the visceral peritoneum. The amebocyte is derived from tissues other than the chloragogen.

2. A Stage I sperm was demonstrated for Limnodrilus hoffmeisteri. The morula-like structure contains approximately 25 developing spermatocytes.

3. Differential cell counts were analyzed using an ANOVA for laboratory maintained worms. The analysis showed significant changes in cell numbers for the chloragogen and amebocytes over the period of time used for the experiment. The Stage I sperm cell counts did not show any significant changes during this period of time. Homogeniety test showed high variance of the data, and the results should be viewed cautiously.

4. Differential cell counts were analyzed using an ANOVA for field studies involving seasonal changes in cell numbers. All three types showed significant changes in cell numbers for the seasons of the year. The changes in cell numbers for Stage I sperm suggested a winter/spring peak in reproductive activity for Limnodrilus hoffmeisteri that were collected in the Kalamazoo, Michigan area. The occurrence of this cell in reduced numbers at other times of the year suggested that the worm is capable of reproducing on a year-round basis.

5. Differential cell counts were analyzed from worms that were

exposed to three levels (7,000 R, 3,000 R, 1,000 R) of X-irradiation. The effect of the radiation on the cell counts was not related to the level of radiation administered. Cell counts changed significantly over the post-irradiation time series used in this experiment for the chloragogen and the Stage I sperm. The amebocytes were too few in number to be analyzed.

6. Morphological examination of the irradiated worms' Stage I sperm showed complete deterioration and subsequent elimination of sperm products by the end of the radiation experiment. The worms appear to have been sterilized by the radiation, but since this study did not examine the entire life cycle of this worm it cannot be concluded that population death would occur in the next generation.

7. The chloragogen cells were intensely granulated by the end of the radiation experiment. The visceral peritoneum became ladened with the heavily granulated chloragogen cells, and this area appeared to be almost totally black from the cells. The chloragogen appeared to be absorbing the deteriorating sperm packets, and this activity may suggest a phagocytic role for these cells.

8. The amebocytes were so few in number during the radiation experiment that an examination of these cells could not be accomplished. It is suggested that these cells may have a role in regeneration and wound healing, and that these cells may be found in the coelom only during those time periods when the worm is regenerating new segments or in the process of wound healing.

TABLE 1

.01 PER CENT KNOP'S SOLUTION

57.1 mg/liter	$Ca(NO_3)_2$
21.4 mg/liter	KNO_3
21.4 mg/liter	$MgSO_4$

WRIGHT'S STAIN

REAGENTS: 1. 12.5 gm Wright's stain (Certified)
2. 3,000 ml Acetone free Methyl Alcohol

PROCEDURE: Place the dye in a gallon bottle. Add 600 ml alcohol, shake well. Add 600 ml alcohol, shake again. Add the remaining amount of alcohol and shake until stain is well mixed. Shake well periodically during the 6 week maturation period. FILTER BEFORE USING.

WRIGHT'S BUFFER

KH_2PO_4	6.63 gm
Na_2PO_4	3.2 gm
Distilled Water	2,000 ml

TABLE 2

STOCK GIEMSA STAIN

REAGENTS:
1. Giemsa stain (Certified)
2. 50 ml methyl alcohol (acetone free)
3. 50 ml glycerin (neutral; from freshly opened bottle)

PROCEDURE: Grind powder in mortar before weighing. Weigh out 600 mg Giemsa stain. Grind stain powder with part of the neutral glycerin in a mortar. Pour the top third off into a chemically clean flask. Add more glycerin and grind again. Repeat until most of the powder has been mixed and poured into the flask. Stopper the flask with a cotton plug; place a heavy paper cap over the opening and bind with a rubber band. Place in a water bath set at 55°C to 60°C, and allow to stand for 2 hours. Shake gently at 1/2 hour intervals. After 2 hours remove the glycerin and stain powder from the bath. Allow to come to room temperature. Add the measured alcohol and the washings from the mortar and shake well. It is preferable to age the stain 2 weeks before use. Shake bottle before pipetting off stain for working solution. NEVER put a wet pipette in the stock stain.

WORKING GIEMSA STAIN

1. Prepare working Giemsa stain each time while slides are drying from Wright's stain. (Giemsa can be used several weeks after a slide has been stained with Wright's.)
2. Put 20 ml distilled water in a flask.
3. Add 20 drops of stock Giemsa, one drop at a time, from a 1 ml pipette.
4. Shake flask gently after adding each drop.

The above directions are adequate for a Coplin jar. Larger amounts are prepared in the same manner: 1 drop of stock/ml of distilled water. A large staining dish requires approximately 300 ml stain.

TABLE 3

Per Cent Composition of *Limnodrilus hoffmeisteri* with other Tubificidae in Portage Creek, Kalamazoo, Michigan October, 1970 through June, 1971

Sample Number	Sample Date	% *Limnodrilus hoffmeisteri*	% Other Tubificidae
1	October 10, 1970	72	28
2	December 12, 1970	32	68
3	February 4, 1971	58	42
4	April 8, 1971	29	71
5	June 4, 1971	31	69

TABLE 4

Raw Data for differential cell counts of *Limnodrilus hoffmeisteri* under laboratory conditions December 8, 1969 through July 29, 1970

Date	Chloragogen	Amebocytes	Stage I Sperm
12-8-69	75	17	8
12-8-69	75	14	11
12-8-69	73	14	13
12-8-69	28	6	66
12-9-69	92	7	1
12-9-69	50	44	6
12-9-69	55	23	22
2-5-70	89	9	2
2-5-70	90	9	1
2-5-70	41	58	1
2-5-70	61	27	12
2-5-70	82	9	9
2-26-70	60	39	1
2-26-70	50	10	40
5-2-70	60	10	30
5-2-70	89	11	0
5-2-70	89	11	0
5-2-70	96	3	1
5-2-70	90	8	2
5-2-70	69	9	22
5-21-70	95	4	1
5-21-70	97	1	2
5-21-70	89	7	4
5-21-70	85	12	3
5-21-70	89	6	5
5-21-70	88	4	8
5-21-70	95	5	0
6-7-70	99	1	0
6-7-70	72	5	23
6-7-70	83	16	1
6-30-70	86	12	2
6-30-70	74	24	2
6-30-70	69	22	9
6-30-70	82	11	7
6-30-70	84	10	6
7-6-70	90	2	8
7-6-70	92	4	4
7-6-70	95	1	4
7-6-70	85	5	10
7-6-70	88	7	5

Table 4 continued

Date	Chloragogen	Amebocytes	Stage I Sperm
7-23-70	67	12	21
7-23-70	74	5	21
7-23-70	49	0	51
7-23-70	80	2	18
7-23-70	96	2	2
7-23-70	88	5	7
7-23-70	83	7	10
7-23-70	97	1	2
7-23-70	94	6	0
7-23-70	86	4	10
7-29-70	48	7	45
7-29-70	59	1	40
7-29-70	95	5	0
7-29-70	60	1	39

TABLE 5

One Way Analysis of Variance for differences in chloragogen cells for laboratory maintained Limnodrilus hoffmeisteri December 8, 1969 through July 29, 1970

Source	S.S.	df	Mean Square	F	Significant at
Between Times (Cell Numbers)	5498.46090	10	549.84609	2.48	< .05
Within Time (Worm Variation)	9520.37111	43	221.40398		
Total	15018.83190	53			

$F_{10,43}$ = 2.08 at the .05 probability level

TABLE 6

One Way Analysis of Variance for differences in ameboid cells for laboratory maintained Limnodrilus hoffmeisteri December 8, 1969 through July 29, 1970

Source	S.S.	df	Mean Square	F	Significant at
Between Times (Cell Numbers)	2873.00225	10	287.30023	3.45	< .05
Within Time (Worm Variation)	3575.83106	43	83.15886		
Total	6448.83335	53			

$F_{10,43}$ = 2.08 at the .05 probability level

TABLE 7

One Way Analysis of Variance for differences in Stage I sperm for laboratory maintained Limnodrilus hoffmeisteri December 8, 1969 through July 29, 1970

Source	S.S.	df	Mean Square	F	Significant at
Between Times (Cell Numbers)	3533.70477	10	353.37048	1.88	> .05
Within Time (Worm Variation)	8099.62861	43	188.36346		
Total	11633.33340	53			

$F_{10,43} = 2.08$ at the .05 probability level

TABLE 8

Raw Data for differential cell counts of *Limnodrilus hoffmeisteri* under field conditions
October 10, 1970 through June 4, 1971

Date	Chloragogen	Amebocytes	Stage I Sperm
10-10-70	99	1	0
	95	5	0
	74	4	22
	99	1	0
	95	4	1
	85	10	5
	93	7	0
	98	2	0
	87	13	0
	99	1	0
	98	2	0
	97	3	0
	94	6	0
	100	0	0
	94	6	0
	100	0	0
	100	0	0
	100	0	0
	99	1	0
	97	3	0
	95	5	0
	86	7	7
	85	15	0
	96	4	0
	90	10	0
	95	5	0
	99	1	0
	100	0	0
	95	5	0
	63	1	36
	96	4	0
	100	0	0
	98	2	0
	100	0	0
	96	4	0
	100	0	0
	95	0	5
	99	0	1
	100	0	0
	97	0	3

Table 8 continued

Date	Chloragogen	Amebocytes	Stage I Sperm
10-10-70	100	0	0
	95	4	1
	100	0	0
	100	0	0
	100	0	0
	100	0	0
	90	10	0
	100	0	0
	100	0	0
	92	8	0
	100	0	0
	100	0	0
	100	0	0
	90	0	10
	100	0	0
	54	0	46
	46	0	54
	100	0	0
	100	0	0
	96	0	4
	72	0	28
	100	0	0
	100	0	0
	29	0	71
	100	0	0
	100	0	0
	100	0	0
	100	0	0
	96	0	4
	100	0	0
	100	0	0
	100	0	0
12-12-70	100	0	0
	100	0	0
	100	0	0
	98	0	2
	68	0	32
	29	0	71
	16	0	84
	100	0	0
	100	0	0
	100	0	0
	100	0	0
	100	0	0
	100	0	0
	5	0	95
	48	0	52

Table 8 continued

Date	Chloragogen	Amebocytes	Stage I Sperm
12-12-70	100	0	0
	81	0	19
	80	0	20
	100	0	0
	100	0	0
	44	0	56
	94	0	6
	100	0	0
	74	0	26
	100	0	0
	71	0	29
	0	0	100
	100	0	0
	100	0	0
	0	0	100
	100	0	0
	47	0	53
2-4-71	59	41	0
	46	43	11
	80	20	0
	73	27	0
	71	29	0
	83	12	0
	38	15	47
	75	21	4
	28	15	57
	30	70	0
	24	25	51
	29	10	61
	16	12	72
	42	33	25
	35	20	45
	65	14	21
	85	15	0
	58	41	1
	88	12	0
	47	10	43
	53	2	45
	48	23	29
	79	21	0
	72	25	3
	50	7	43
	66	20	14
	88	12	0
	90	7	3
	90	7	3
	91	9	0

Table 8 continued

Date	Chloragogen	Amebocytes	Stage I Sperm
2-4-71	94	6	0
	22	7	71
	89	10	1
	92	6	2
	36	13	51
	88	6	6
	99	0	1
	81	6	13
	89	10	1
	62	1	37
	70	2	28
	98	0	2
	97	0	3
	57	2	41
	48	1	51
	92	8	0
	73	6	21
	97	3	0
	95	5	0
	21	0	79
	0	0	100
	96	4	0
	93	7	0
	97	3	0
	98	1	1
	100	0	0
	97	3	0
	5	0	95
4-8-71	82	2	16
	100	0	0
	100	0	0
	48	2	50
	85	1	14
	72	16	12
	60	8	32
	75	5	20
	100	0	0
	66	3	31
	57	7	36
	100	0	0
	72	1	27
	56	3	41
	96	3	1
	97	0	3
	85	0	15
	92	0	8
	100	0	0

Table 8 continued

Date	Chloragogen	Amebocytes	Stage I Sperm
4-8-71	93	7	3
	53	0	47
	98	0	2
	56	6	38
	87	0	13
	73	1	26
	100	0	0
	74	1	25
	100	0	0
	100	0	0
6-4-71	60	4	36
	25	0	75
	93	0	7
	100	0	0
	97	3	0
	100	0	0
	99	0	1
	39	2	59
	76	0	24
	98	0	2
	100	0	0
	99	0	1
	99	0	1
	100	0	0
	100	0	0
	17	0	83
	100	0	0
	35	0	65
	33	4	63
	100	0	0
	100	0	0
	100	0	0
	100	0	0
	95	5	0
	99	0	1
	92	8	0
	96	0	4
	100	0	0
	100	0	0
	100	0	0
	84	0	16

TABLE 9

One Way Analysis of Variance for Seasonal differences in chloragogen cells of Limnodrilus hoffmeisteri October 10, 1970 through June 4, 1971

Source	S.S.	df	Mean Square	F	Significant at
Between Times (Cell Numbers)	24103.39060	4	6025.84764	10.82	< .05
Within Time (Worm Variation)	120827.82900	217			
Total	144931.22000	221			

$F_{4,217}$ = 2.37 at the .05 probability level

TABLE 10

One Way Analysis of Variance for Seasonal differences in ameboid cells of Limnodrilus hoffmeisteri October 10, 1970 through June 4, 1971

Source	S.S.	df	Mean Square	F	Significant at
Between Times (Cell Numbers)	5387.28401	4	1346.82100	25.48	< .05
Within Time (Worm Variation)	11468.68460	217	52.85108		
Total	16855.96850	221			

$F_{4,217}$ = 2.37 at the .05 probability level

TABLE 11

One Way Analysis of Variance for Seasonal differences in Stage I sperm of Limnodrilus hoffmeisteri October 10, 1970 through June 4, 1971

Source	S.S.	df	Mean Square	F	Significant at
Between Times (Cell Numbers)	12282.23880	4	3070.55969	5.67	<.05
Within Time (Worm Variation)	117588.37800	217	541.88193		
Total	129870.61800	221			

$F_{4,217} = 2.37$ at the .05 probability level

TABLE 12

Raw Data of the differential cell counts for *Limnodrilus hoffmeisteri* after exposure to X-irradiation

Time Series	Hours Post Irradiation	Radiation Level (R)	Chloragogen	Amebocytes	Stage I Sperm
1	11	1,000	46	0	54
			100	0	0
			71	0	29
			53	0	47
			22	0	78
			38	0	62
			35	0	65
			41	0	59
			34	0	66
			57	0	43
2	25	1,000	67	0	33
			31	0	69
			58	0	42
			98	0	2
			75	0	25
			80	0	20
			91	0	9
			88	0	12
			49	0	51
3	55	1,000	26	0	74
			7	0	93
			95	0	5
			56	0	44
			76	0	24
			17	0	83
			64	0	36
			29	0	71
			100	0	0
4	78	1,000	74	0	26
			96	0	4
			93	0	7
			87	0	13
			92	0	8
			82	0	18
			82	0	18
			60	0	40
			32	0	68
			76	0	24
5	122	1,000	93	0	7
			69	0	31
			43	0	57
			57	0	43

Table 12 (Continued)

Time Series	Hours Post Irradiation	Radiation Level (R)	Chloragogen	Amebocytes	Stage I Sperm
5	122	1,000	100	0	0
			100	0	0
			36	0	64
			37	0	63
			99	0	1
			71	0	29
6	170	1,000	81	0	19
			70	0	30
			52	0	48
			65	0	35
			93	0	7
			73	0	27
			89	0	11
			89	0	11
			76	0	24
			43	0	57
7	338	1,000	100	0	0
			98	0	2
			90	0	10
			100	0	0
			91	0	9
			100	0	0
			61	0	39
			93	0	7
			98	0	2
			100	0	0
8	605	1,000	100	0	0
			87	0	13
			100	0	0
			99	0	1
			90	0	10
			89	0	11
			96	0	4
			97	0	3
			96	0	4
			99	0	1
			75	0	25
9	768	1,000	66	0	34
			100	0	0
			100	0	0
			95	0	5
			89	0	11
			100	0	0
			100	0	0
			100	0	0
			89	0	11

Table 12 (Continued)

Time Series	Hours Post Irradiation	Radiation Level (R)	Chloragogen	Amebocytes	Stage I Sperm
9	768	1,000	100	0	0
1	11	3,000	62	0	38
			62	1	37
			22	0	78
			100	0	0
			64	0	36
2	25	3,000	87	2	11
			41	2	57
			60	0	40
			63	0	37
			56	0	44
			87	0	13
			80	0	20
			33	0	67
			100	0	0
			88	0	12
3	55	3,000	31	0	69
			32	0	68
			83	0	17
			39	0	61
			65	0	35
			100	0	0
			47	0	53
			95	0	5
			52	0	48
			4	0	96
4	78	3,000	47	0	53
			100	0	0
			39	0	61
			54	0	46
			100	0	0
			16	0	84
			90	0	10
			18	0	82
5	122	3,000	73	0	27
			69	0	31
			98	0	2
			84	0	16
			100	0	0
			69	0	31
			70	0	30
			50	0	50
			99	0	1
			100	0	0
6	170	3,000	90	0	10
			92	0	8

Table 12 (Continued)

Time Series	Hours Post Irradiation	Radiation Level (R)	Chloragogen	Amebocytes	Stage I Sperm
6	170	3,000	99	0	8
			96	0	4
			100	0	0
			98	0	2
			92	0	8
			100	0	0
			100	0	0
			96	0	4
7	338	3,000	100	0	0
			100	0	0
			100	0	0
			100	0	0
			88	0	12
			89	0	11
			39	0	61
			100	0	0
			67	33	0
8	605	3,000	100	0	0
			100	0	0
			96	0	4
			100	0	0
			100	0	0
			98	0	2
			92	0	8
			100	0	0
			100	0	0
			100	0	0
9	768	3,000	100	0	0
			100	0	0
			100	0	0
			100	0	0
			100	0	0
			100	0	0
			100	0	0
			100	0	0
			100	0	0
			100	0	0
1	11	7,000	62	0	38
			10	1	89
			100	0	0
			21	0	79
			13	0	87
			100	0	0
			95	0	5
			80	0	20

Table 12 (Continued)

Time Series	Hours Post Irradiation	Radiation Level (R)	Chloragogen	Amebocytes	Stage I Sperm
1	11	7,000	98	1	1
			65	0	35
2	25	7,000	70	0	30
			65	0	35
			89	11	0
			60	0	40
			88	12	0
			24	1	75
			83	4	13
			100	0	0
			76	0	24
			95	0	5
3	55	7,000	52	0	48
			97	3	0
			37	0	63
			98	0	2
			86	0	14
			100	0	0
			21	0	79
			93	0	7
			100	0	0
4	78	7,000	62	0	38
			47	0	53
			94	2	4
			64	0	36
			67	0	33
			90	1	9
			59	0	41
			31	1	68
			81	0	19
			87	0	12
5	122	7,000	50	0	50
			98	0	2
			78	0	22
			77	3	20
			100	0	0
			14	0	86
			94	0	6
			42	0	58
			48	0	52
			92	0	8
6	170	7,000	88	0	12
			100	0	0
			100	0	0
			96	0	4
			62	0	38

Table 12 (Continued)

Time Series	Hours Post Irradiation	Radiation Level (R)	Chloragogen	Amebocytes	Stage I Sperm
6	170	7,000	50	0	50
			68	2	30
			100	0	0
			86	0	14
			60	0	40
7	338	7,000	100	0	0
			100	0	0
			100	0	0
			100	0	0
			100	0	0
			66	0	34
			100	0	0
			100	0	0
			100	0	0
			100	0	0
8	605	7,000	100	0	0
			83	0	17
			100	0	0
			100	0	0
			100	0	0
			100	0	0
			100	0	0
			100	0	0
			100	0	0
9	768	7,000	100	0	0
			100	0	0
			100	0	0
			100	0	0
			100	0	0
			100	0	0
			97	0	3
			100	0	0
			100	0	0
			100	0	0
1	11	CONTROL 1	88	12	0
			67	18	0
			85	15	0
			92	6	2
			78	11	11
			96	1	3
			21	2	77
			92	1	7
			97	3	0
			31	0	69
2	25	CONTROL 1	98	0	2
			100	0	0

Table 12 (Continued)

Time Series	Hours Post Irradiation	Radiation Level (R)	Chloragogen	Amebocytes	Stage I Sperm
2	25	CONTROL 1	98	0	2
			55	0	45
			98	0	2
			29	0	71
			7	0	93
			75	0	25
			74	0	26
			86	0	14
3	55	CONTROL 1	19	0	81
			0	0	100
			89	0	11
			56	0	44
			14	0	86
			100	0	0
			52	0	48
			32	0	68
4	78	CONTROL 1	87	0	13
			99	0	1
			44	0	56
			100	0	0
			22	0	78
			96	0	4
			90	0	10
			73	0	27
			32	0	68
5	122	CONTROL 1	99	0	1
			27	0	73
			83	0	17
			100	0	0
			100	0	0
			99	0	1
			98	0	2
			49	0	51
			67	0	33
			46	0	54
6	170	CONTROL 1	100	0	0
			100	0	0
			74	0	26
			73	0	27
			69	0	31
			25	0	75
			82	0	18
			79	0	21
			92	0	8
			70	0	30

Table 12 (Continued)

Time Series	Hours Post Irradiation	Radiation Level (R)	Chloragogen	Amebocytes	Stage I Sperm
7	338	CONTROL 1	100	0	0
			100	0	0
			97	0	3
			100	0	0
			89	0	11
			82	0	18
			33	0	67
			98	0	2
			60	0	40
			10	0	90
8	605	CONTROL 1	7	0	93
			78	0	22
			98	0	2
			91	0	9
9	768	CONTROL 1	81	0	19
			92	0	8
			92	0	8
			100	0	0
			96	0	4
			77	0	23
			100	0	0
			99	0	1
			100	0	0
			100	0	0
1	11	CONTROL 2	90	0	10
			84	0	16
			32	0	68
			12	0	88
			27	0	73
			32	0	68
			17	0	83
			74	0	26
2	25	CONTROL 2	76	0	24
			79	0	21
			100	0	0
			12	0	88
			70	0	30
			98	0	2
			80	0	20
			64	0	36
			56	0	44
3	55	CONTROL 2	88	0	12
			52	0	48
			64	0	36
			25	0	75
			75	0	25

Table 12 (Continued)

Time Series	Hours Post Irradiation	Radiation Level (R)	Chloragogen	Amebocytes	Stage I Sperm
3	55	CONTROL 2	66	0	34
			5	0	95
			99	0	1
			41	0	59
			81	0	19
4	78	CONTROL 2	91	0	9
			66	0	34
			70	0	30
			100	0	0
			72	0	28
			94	0	6
			86	0	14
			47	0	53
			91	0	9
			79	0	21
5	122	CONTROL 2	85	0	15
			41	0	59
			57	0	43
			100	0	0
			100	0	0
			98	0	2
			95	0	5
			27	0	73
			59	0	41
			26	0	74
6	170	CONTROL 2	52	0	48
			100	0	0
			58	0	42
			92	0	8
			59	0	41
			92	0	8
			47	0	53
7	338	CONTROL 2	69	0	31
			45	0	55
			100	0	0
			98	0	2
			70	0	30
			100	0	0
			91	0	9
			85	0	15
			59	0	41
			100	0	0
8	605	CONTROL 2	98	0	2
			99	0	1
			86	0	14
			100	0	0

Table 12 (Continued)

Time Series	Hours Post Irradiation	Radiation Level (R)	Chloragogen	Amebocytes	Stage I Sperm
8	605	CONTROL 2	75	0	25
			97	0	3
			95	0	5
			57	0	43
			76	0	24
			83	0	17
9	768	CONTROL 2	97	0	3
			80	0	20
			99	0	1
			99	0	1
			84	0	16
			96	0	4
			99	0	1
			92	0	8
			48	0	52
			90	0	10

TABLE 13

Two Way Analysis of Variance using an Arcsin transformation for differences in chloragogen cells for Limnodrilus hoffmeisteri after exposure to X-irradiation
Comparison with Control 1

Source	S.S.	df	Mean Square	F	Significant at
Cells	11.16	35			
1* eliminating 2**	0.70	3	0.23	2.21	> .05
2 eliminating 1	7.95	8	0.99	9.48	< .05
1 by 2	2.52	24	0.10		
Total	11.16	35			

*1: Levels of Radiation (7,000 R, 3,000 R, 1,000 R)
**2: Time Series 1 through 9

*$F_{3,24}$ = 3.01 at the .05 probability level

**$F_{8,24}$ = 2.36 at the .05 probability level

TABLE 14

Two Way Analysis of Variance using an Arcsin transformation for differences in Stage I sperm for Limnodrilus hoffmeisteri after exposure to X-irradiation
Comparison with Control 1

Source	S.S.	df	Mean Square	F	Significant at
Cells	3.31	35			
1* eliminating 2**	0.13	3	0.04	1.16	> .05
2 eliminating 1	2.24	8	0.28	7.24	< .05
1 by 2	0.93	24	0.04		
Total	3.31	35			

*1: Levels of Radiation (7,000 R, 3,000 R, 1,000 R)
**2: Time Series 1 through 9

*$F_{3,24}$ = 3.01 at the .05 probability level

**$F_{8,24}$ = 2.36 at the .05 probability level

TABLE 15

Two Way Analysis of Variance using an Arcsin transformation for differences in chloragogen cells for Limnodrilus hoffmeisteri after exposure to X-irradiation
Comparison with Control 2

Source	S.S.	df	Mean Square	F	Significant at
Cells	11.49	35			
1* eliminating 2**	0.78	3	0.26	3.38	< .05
2 eliminating 1	8.87	8	1.11	14.44	< .05
1 by 2	1.84	24	0.08		
Total	11.49	35			

*1: Levels of Radiation (7,000 R, 3,000 R, 1,000 R)
**2: Time Series 1 through 9

*$F_{3,24}$ = 3.01 at the .05 probability level

**$F_{8,24}$ = 2.36 at the .05 probability level

TABLE 16

Two Way Analysis of Variance using an Arcsin transformation for differences in Stage I sperm for Limnodrilus hoffmeisteri after exposure to X-irradiation
Comparison with Control 2

Source	S.S.	df	Mean Square	F	Significant at
Cells	3.58	35			
1* eliminating 2**	0.19	3	0.06	2.64	> .05
2 eliminating 1	2.82	8	0.35	14.76	< .05
1 by 2	0.57	24	0.02		
Total	3.58	35			

*1: Levels of Radiation (7,000 R, 3,000 R, 1,000 R)
**2: Time Series 1 through 9

*$F_{3,24}$ = 3.01 at the .05 probability level

**$F_{8,24}$ = 2.36 at the .05 probability level

TABLE 17

Two Way Analysis of Variance using an Arcsin transformation for comparison of Controls 1 and 2 used in the radiation experiment Differences of chloragogen cells of Limnodrilus hoffmeisteri

Source	S.S.	df	Mean Square	F	Significant at
Cells	2.20	17			
1* eliminating 2**	0.00	1	0.00	0.04	> .05
2 eliminating 1	1.63	8	0.20	2.84	> .05
1 by 2	0.57	8	0.07		
Total	2.20	17			

*1: Comparison of Controls 1 and 2
**2: Time Series 1 through 9

*$F_{1,8}$ = 5.32 at the .05 probability level

**$F_{8,8}$ = 3.44 at the .05 probability level

TABLE 18

Two Way Analysis of Variance using an Arcsin transformation for comparison of Controls 1 and 2 used in the radiation experiment Differences of Stage I sperm of Limnodrilus hoffmeisteri

Source	S.S.	df	Mean Square	F	Significant at
Cells	1.13	17			
1* eliminating 2**	0.01	1	0.01	0.13	> .05
2 eliminating 1	0.70	8	0.09	1.67	> .05
1 by 2	0.42	8	0.05		
Total	1.13	17			

*1: Comparison of Controls 1 and 2
**2: Time Series 1 through 9

*$F_{1,8}$ = 5.32 at the .05 probability level

**$F_{8,8}$ = 3.44 at the .05 probability level

Line Separation Technique Explanation
for Tables 19 through 25

C A D B G E F

The line separation technique schematically summarizes the Two Sample T's Test for variance used in analysing differential cell counts for irradiated Limnodrilus hoffmeisteri. The procedure for illustrating the line separations is to place the means of a given cell count for a particular time series in ascending order and connect related time series mean cell numbers by drawing a line under the related groups. In the above example, the time series underlined by a common line do not differ from each other; time series not underlined by a common line do differ. Thus, time series F differs from time series C, A, and D, but time series F does not differ from time series E, G, and B. Similarly time series E differs from C, A, and D but does not differ from B, G, and F. All levels of significance are maintained at .05 level. (From Winer, 1971)

Hours Post-Irradiation for the Time Series Used
in This Study for Tables 19 through 25

Time Series	Hours Post Irradiation
1	11
2	25
3	55
4	78
5	122
6	170
7	338
8	605
9	768

TABLE 19

Two Sample T's Test for Variance - Line Separation Summary for Differential Cell Counts of Limnodrilus hoffmeisteri after exposure to 7,000 R.*

CHLORAGOGEN

Time Series:	1	4	5	2	3	6	7	8	9
Ordered Mean:	64.4	68.2	69.3	75.0	76.0	81.0	96.6	98.1	99.7

STAGE I SPERM

Time Series:	9	8	7	6	2	3	5	4	1
Ordered Mean:	.3	1.89	3.4	18.8	22.2	23.7	30.4	31.3	35.4

*Time series 6 and 7 are not related. This discrepancy using the Line Separation Technique is referred to in the discussion of this paper.

TABLE 20

Two Sample T's Test for Variance - Line Separation Summary for Differential Cell Counts of Limnodrilus hoffmeisteri after exposure to 3,000 R.

CHLORAGOGEN

Time Series:	3	4	1	2	5	7	6	8	9*
Ordered Mean:	54.8	58.0	63.0	69.5	81.2	87.0	96.3	98.6	100.0

STAGE I SPERM

Time Series:	9*	8	6	7	5	2	1	4	3
Ordered Mean:	0.0	1.4	3.7	9.3	18.8	30.1	37.8	42.0	45.2

*A line is not drawn under time series 9 because the test will calculate means that are greater than 0 and less than 100.

TABLE 21

Two Sample T's Test for Variance - Line Separation Summary for Differential Cell Counts of Limnodrilus hoffmeisteri after exposure to 1,000 R.

CHLORAGOGEN

Time Series:	1	3	5	2	6	4	8	7	9
Ordered Mean:	49.7	52.2	70.5	70.8	73.1	77.4	92.8	93.1	93.9

STAGE I SPERM

Time Series:	9	7	8	4	6	2	5	3	1
Ordered Mean:	6.1	6.9	7.2	22.6	26.9	29.2	29.5	47.8	50.3

TABLE 22

Two Sample T's Test for Variance - Line Separation Summary for Differential Cell Counts of Limnodrilus hoffmeisteri.
CONTROL 1

CHLORAGOGEN

Time Series:	3	8	4	2	1	6	5	7	9
Ordered Mean:	51.3	68.5	71.4	70.0	74.4	76.4	76.8	76.9	93.7

STAGE I SPERM

Time Series:	9	1	7	5	6	2	4	8	3
Ordered Mean:	6.3	18.4	23.1	23.2	23.6	27.8	28.6	31.5	48.7

TABLE 23

Two Sample T's Test for Variance - Line Separation Summary for Differential Cell Counts of Limnodrilus hoffmeisteri.
CONTROL 2

CHLORAGOGEN

Time Series:	1	3	5	2	6	4	7	8	9
Ordered Mean:	46.0	59.6	68.8	70.6	71.4	79.6	81.7	86.6	88.4

STAGE I SPERM

Time Series:	9	8	7	4	6	2	5	3	1
Ordered Mean:	11.6	13.4	18.3	20.4	28.6	29.4	30.9	40.4	54.0

TABLE 24

Two Sample T's Test for Variance - Line Separation Summary for Differential Cell Counts of Limnodrilus hoffmeisteri. Control 1 compared with Early Winter Seasonal Data (December 12, 1970)

STAGE I SPERM

Time Series:	9	1	7	5	10*	6	2	4	8	3
Ordered Mean:	6.3	18.4	23.1	23.2	23.3	23.6	27.8	28.6	31.5	48.7

*10 represents the early winter data (December 12, 1970) for differential cell counts.

TABLE 25

Two Sample T's Test for Variance - Line Separation Summary for Differential Cell Counts of Limnodrilus hoffmeisteri. Control 2 compared with Early Winter Seasonal Data (December 12, 1970)

STAGE I SPERM

Time Series:	9	8	7	4	10*	6	2	5	3	1
Ordered Mean:	11.6	13.4	18.3	20.4	23.3	28.6	29.4	30.9	40.4	54.0

*10 represents the early winter data (December 12, 1970) for differential cell counts.

Figure 1

The environmental chamber and X-irradiated cultures of *Limnodrilus hoffmeisteri*

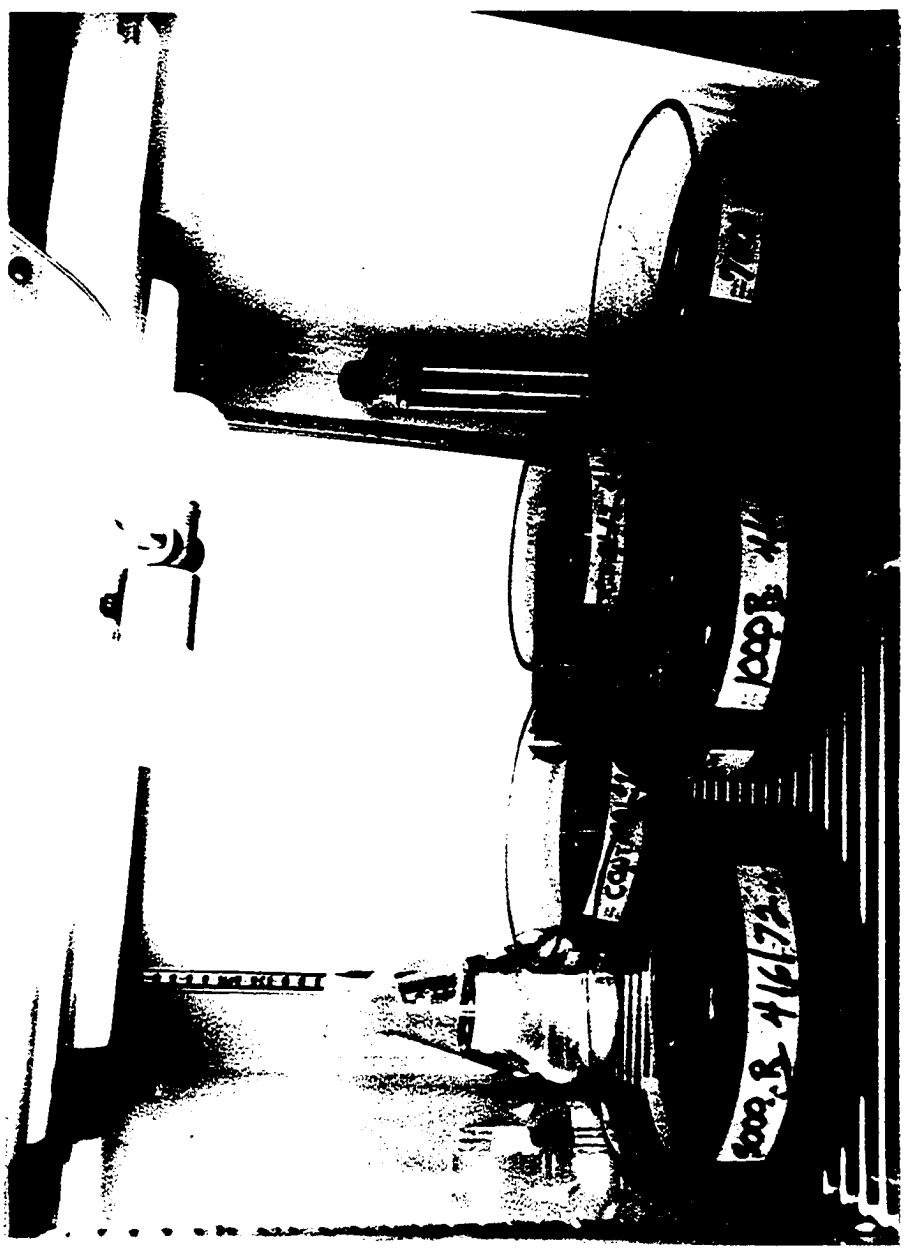


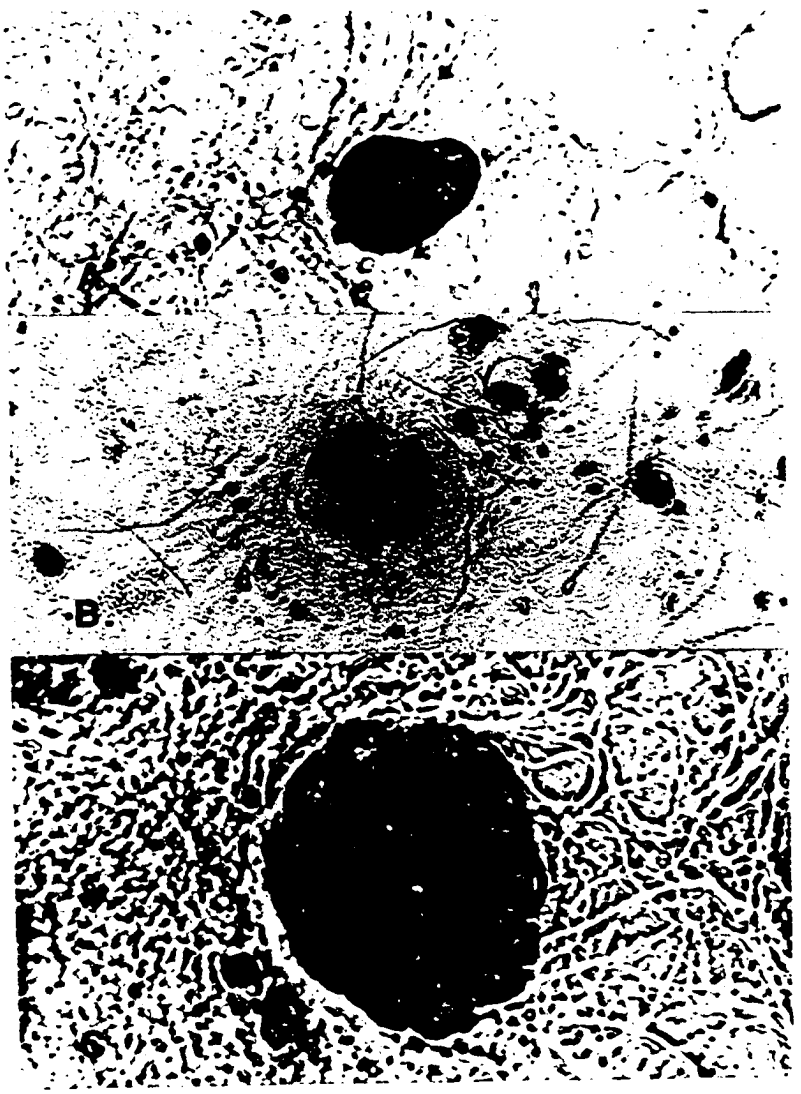
Figure 2

Limnodrilus hoffmeisteri, C.S., showing the association of chloragogen cells with the sperm sac

One micron cross-section of Limnodrilus hoffmeisteri taken in the clitellum region. X 338. Gut lumen (gl); gut epithelial tissue (epl); muscle (m); chloragogen cells (ch); penis sheath (ps); blood vessel (bv); sperm sac (ss); coelom (c).

Figure 3

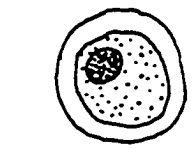
The coelomic cells and Stage I sperm of Limnodrilus hoffmeisteri



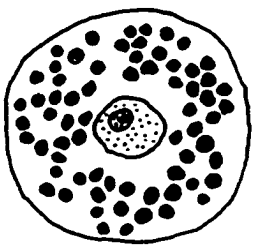
The coelomic cells of Limnodrilus hoffmeisteri consist of an (A.) amebocyte and (B.) the chloragogen cell. The granules found in the chloragogen cell are referred to as chloragosomes. Stage I sperm (C.) forms a morula with approximately 25 developing primary spermatocytes. X 851.

Figure 4

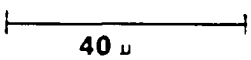
The coelomic cells and Stage I sperm of Limnodrilus hoffmeisteri

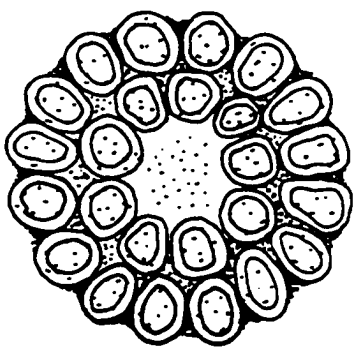


A.



B.



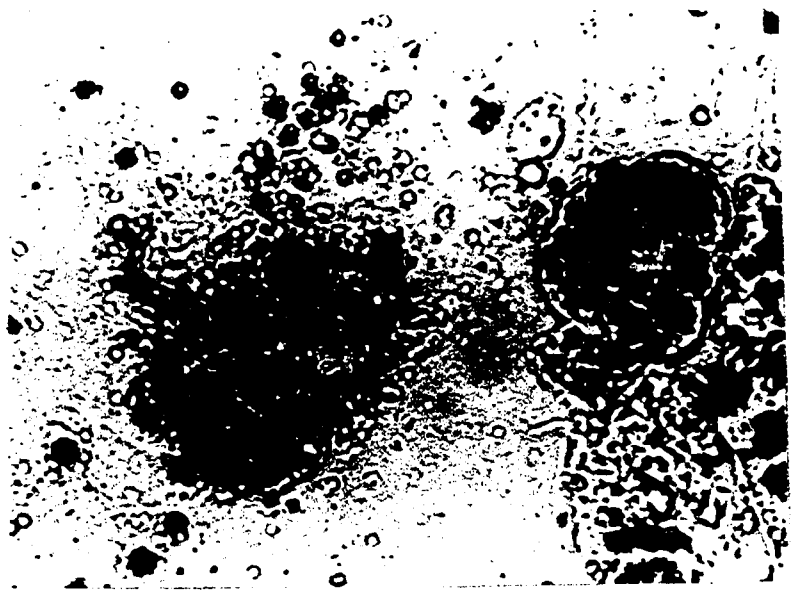


C.

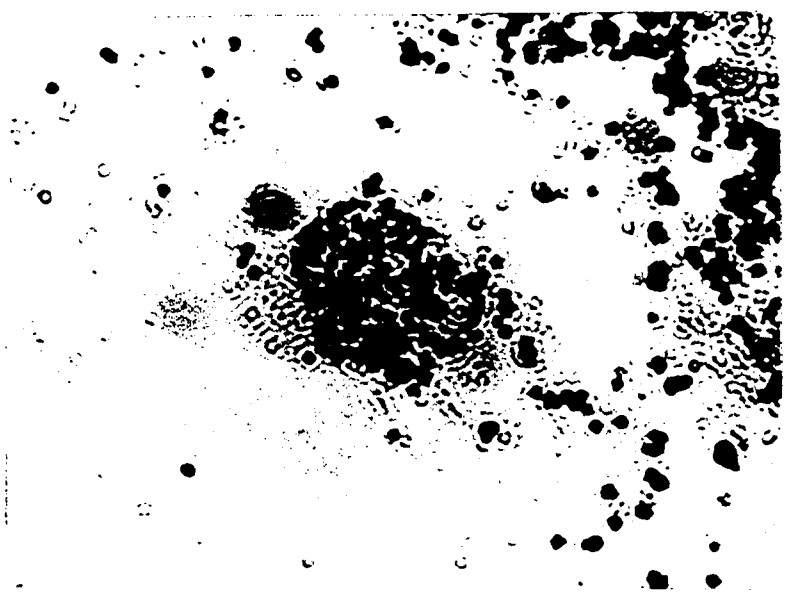
The coelomic cells of Limnodrilus hoffmeisteri consist of an (A.) amebocyte and (B.) the chloragogen cell. The granules found in the chloragogen cell are referred to as chloragosomes. Stage I sperm (C.) forms a morula with approximately 25 developing primary spermatocytes.

Figure 5

Effects of X-irradiation on the chloragogen cells of Limnodrilus hoffmeisteri



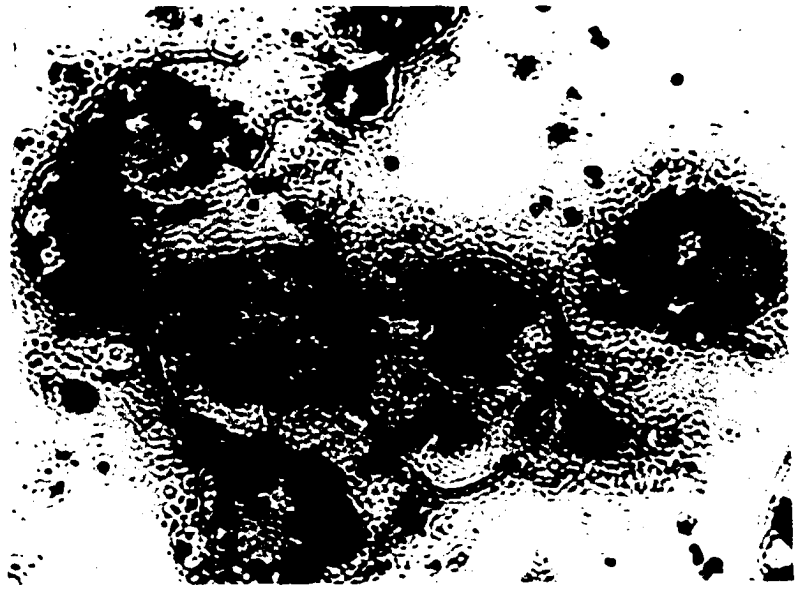
A. Baseline chloragogen cells: This photograph was taken of a Control 1 specimen at time series 9 (768 hours post irradiation). X 851.



B. Baseline chloragogen cell: This photograph was taken of a Control 2 specimen at time series 9 (768 hours post irradiation). X 851.

Figure 6

Effects of X-irradiation on the chloragogen cells of Limnodrilus hoffmeisteri

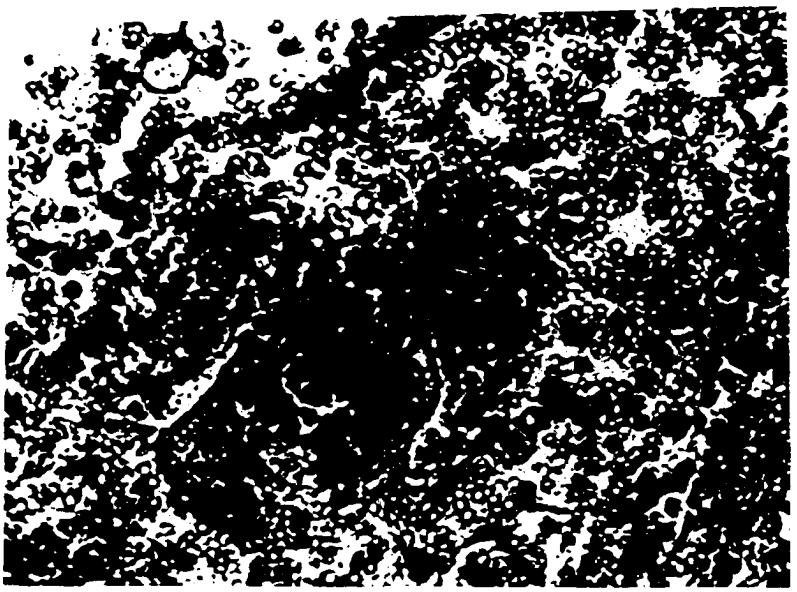


A. Chloragogen cells from worms treated with 7,000 R X-irradiation at time series 3 (55 hours post irradiation): There is little difference between the controls (Figure 5). X 851.

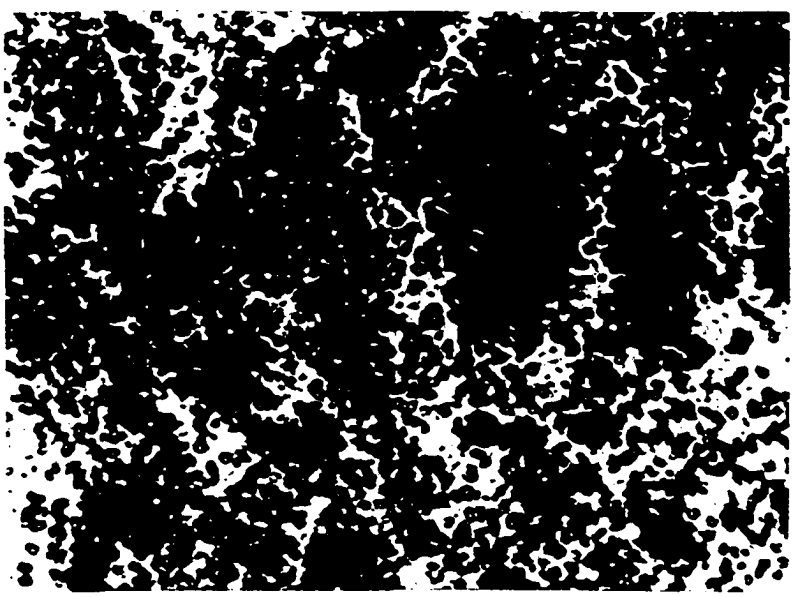
B. Chloragogen cells from worms treated with 7,000 R X-irradiation at time series 6 (170 hours post irradiation): The cells appear to have under gone little change as compared to the controls (Figure 5). X 851.

Figure 7

Effects of X-irradiation on the chloragogen cells of Limnodrilus hoffmeisteri



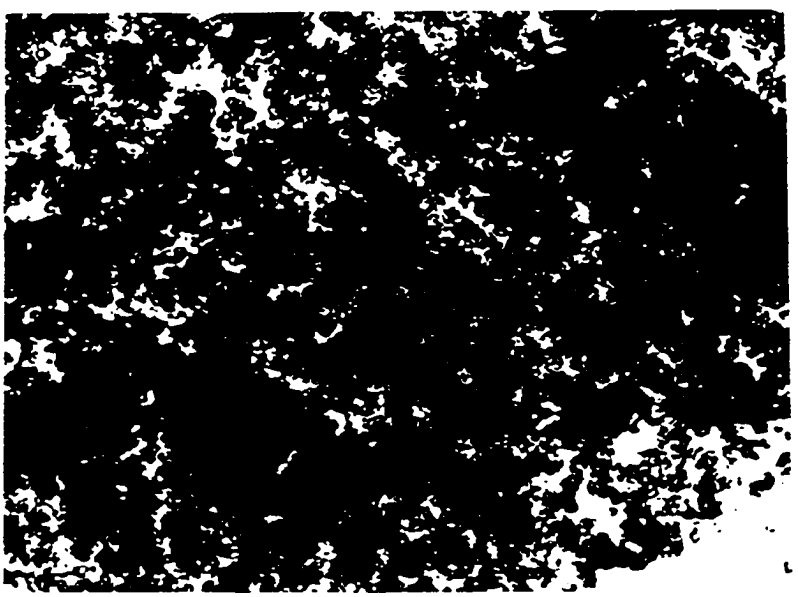
A. Chloragogen cells from worms treated with 7,000 R X-irradiation at time series 7 (338 hours post irradiation): An increase in the granulation of these cells have made individual cell identification difficult. Several nuclei are still visible. These cells are not necrotic. X 851.



B. Chloragogen cells from worms treated with 7,000 R X-irradiation at time series 8 (605 hours post irradiation): The granulation of these cells prevents individual cell definition, and nuclei are difficult to find in this photograph. X 851.

Figure 8

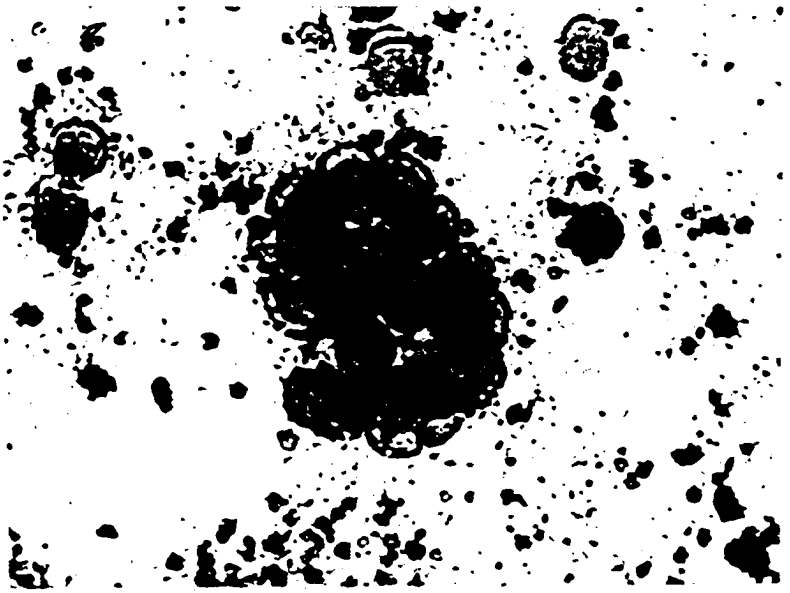
Effects of X-irradiation on the chloragogen cells of Limnodrilus hoffmeisteri



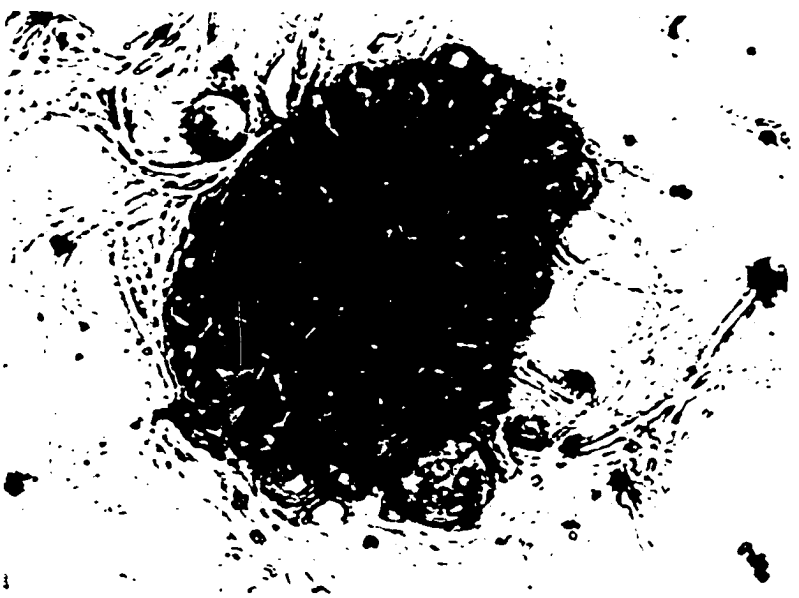
A. Chloragogen cells from worms treated with 7,000 R X-irradiation at time series 9 (768 hours post irradiation). A "charcoal black" effect is easily seen in these cells. Nuclei and cell walls are obscured by the intense granulation. The cells are not necrotic. X 851.

Figure 9

Effects of X-irradiation on Stage I sperm of Limnodrilus hoffmeisteri



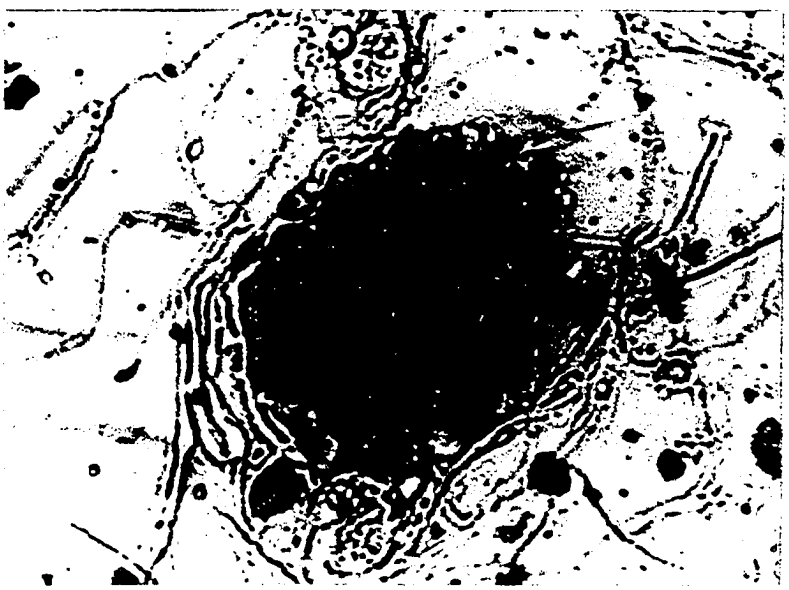
A. Base line Stage I sperm: The normal Stage I sperm was observed in a Control 2 specimen at time series 9 (768 hours post irradiation). X 851.



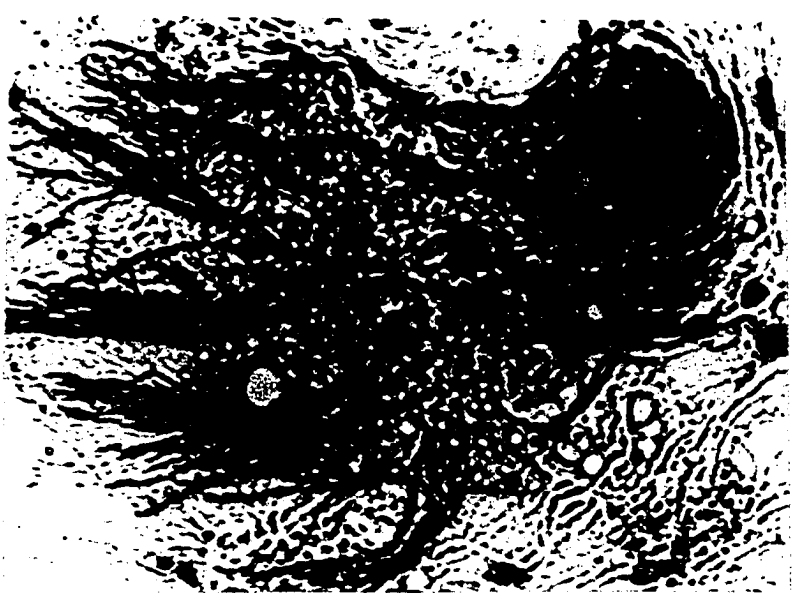
B. Irradiated Stage I sperm: This is a typical form of necrosis. The nuclei are in a pyknotic condition. The cytoplasm is vacuolated and almost indistinguishable from the nuclei. Time series 7 (338 hours post irradiation). X 851.

Figure 10

Effects of X-irradiation on Stage I sperm of Limnodrilus hoffmeisteri



A. Irradiated Stage I sperm: The nuclei and cytoplasm are almost indistinguishable. Vacuolation is seen throughout the morula. Time series 6 (170 hours post irradiation). X 851.



B. Irradiated Advanced Matured sperm: The necrotic condition of this stage is indicated by the intense vacuolation of the morula. The long filaments are mature sperm. Note the chloragogen cell in the upper right corner of the photo. Series 6 (170 hours post irradiation). X 851.

Figure 11

Effects of X-irradiation on mature sperm of Limnodrilus hoffmeisteri

A. Irradiated Sperm: The sperm is shortened and somewhat rod-like. Many small projections can be seen on the outer wall of the sperm. Series 6 (170 hours post irradiation). X 851.

B. Baseline sperm: The normal sperm appears to be a long, smooth, cylindrical structure coming together at a point at either ends. X 851.

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