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Studies on the Nutritional Aspects of Tubificid Worms

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STUDIES ON THE NUTRITIONAL ASPECTS OF TUBIFICID WORMS

(TITLE)

BY

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THESIS

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INTRODUCTION

Tubificidae is a family of aquatic worms belonging to the class Oligochaeta of the phylum Annelida. The worms are reddish-colored, slender, and less than two hundred millimeters in length. The nature of the substrate, the water quality, and the salinity limit the distribution of the species (Brinkhurst and Kennedy, 1962). They normally inhabit the mud or silt of benthic areas of bodies of water. Many members of the family construct tubes in the substrate into which the worm can withdraw; this also serves as a holdfast as the posterior portion of the organism is held away from the substrate into the surrounding environment. The exposed portion of the body is continually waved to and fro apparently in order to facilitate the exchange of carbon dioxide for oxygen across the body wall--only one species of the family has actual gills (Stephenson, 1930). The worms may also be found deeper in the substrate (Brinkhurst and Kennedy, 1965). Most species may be found in slightly to heavily organically-polluted waters, being more abundant there than in relatively clean waters; a few are estuarine and marine. The combined effect of the reduction of predators due to their being less tolerable of polluted conditions and the more favorable conditions for the worms

themselves commonly allows tubificids to be the only metazoan present in polluted waters. While the degree of salinity definitely limits the range of certain species, it has no effect on oxygen consumption when kept within the tolerable range of the organism (Palmer, 1968). The use of particular species or combination of species of tubificids as indicators of pollution had been suggested (Goodnight and Whitley, 1960; Goodnight and Whitley, 1961).

A great deal of work has been done on the taxonomy of the group recently; many genera and species have been revised and redescribed. Distributions, habitats, and life histories have been studied for many species. These preliminary studies must be made, however, before the role of this group may be fully understood in the ecosystem. It has been suggested that the nutrition of the family be investigated. The organism derives nutrition from the benthic ooze containing organic detritus and micro-organisms, especially bacteria. The bacteria may be used directly by the tubificid, being broken down by the enzymes of the gastro-intestinal tract, or retained and utilized in a symbiotic relationship (Brinkhurst, 1970). The bacteria may be kept alive in the gut to aid the worm in the breakdown of the detritus also taken in. It was the purpose of this research, then, to isolate and identify the various species of aerobic bacteria (Schizomycetes, especially Eubacteriales) common to the tubificid gut and from their characteristics to suggest the possible nature of their relationship to the nutritional physiology of the worm.

LITERATURE REVIEW

The taxonomy of the Tubificidae has recently been revised. The family was first described by Michaelesen (1886); Stephenson (1930) updated and revised the family in his volume on the Oligochaeta. Brinkhurst (1960), possibly the most prolific modern writer on the Tubificidae, published a redescription of certain species found in Britain. Brinkhurst (1962) also published A Checklist of British Oligochaeta; a year later (1963a) he revised the checklist, correcting some errors and omissions. Later (1963b), he listed nineteen brackish-water and marine tubificids and created a new genus. He also (1963c) brought together all the descriptions of the tubificids, gave the taxonomic relationships of the worms, included a key to twenty-five species, and gave their geographical distribution. He published (1963d) A Guide to the Identification of British Aquatic Oligochaetes. He found (1963e) a brackish-water species previously unrecorded in Britain--Aktedrilus monospermathecus Knollner, 1935. A year later (1964b), he summarized the taxonomy and biology of the marine worm Tubifex costatus (Claparede); this was the first of a series of papers on the life histories and biology of British tubificids. Brinkhurst then (1965b) concentrated his efforts on the North American tubificids.

Forty-four species were described, twelve new species were added, and their geographical distributions given. Brinkhurst (1965c) reported on the improvement in the biological and chemical conditions of the River Derwent, Derbyshire, England, after a new sewage disposal works and other improvements had been installed. Kennedy (1965) outlined the geographical distribution of seven species of Limnodrilus which were found to have a low tolerance to organic and inorganic pollutants and to high salinities. Kennedy (1966a, 1966b) described the life histories, and systematics of Limnodrilus hoffmeisteri and L. udekemianus. Brinkhurst (1966a) added a supplement to his 1963 publication of taxonomic studies of the tubificids. Brinkhurst (1966b) dealt with the revision of six families of African aquatic oligochaetes, including the tubificids, giving their habits and a key to their identification. He also (1966c) revised the genus Clitello of the marine forms.

Many faunistic surveys concerning the distribution of tubificids have been undertaken. Brinkhurst and Kennedy (1962) recorded fourteen species of aquatic oligochaetes from the Isle of Man. Brinkhurst (1963f) surveyed the aquatic oligochaetes of an Italian lake and constructed a key to their identification. He then (1964a) compared the tubificid fauna of British and European lakes with that of the known distribution of species of the world and discussed the classification of lakes with regard to the tubificids present. Brinkhurst (1964c) summarized the species distribution of lakes, rivers, and saline waters. Roth and Neff (1964) studied the

benthos of Mountain Lake, Virginia, and listed tubificids as being one of the predominant forms. Buckley and Sublette (1964) stated that oligochaetes, presumably tubificids, were the predominant benthic organism in a Louisianian lake in both volume and numbers at each depth sampled. Schaefer and Harrol (1965) gave new distribution records for Branchiura sowerbyi with seasonal and habitat density information. Carr and Hiltunen (1965) sampled the benthos of western Lake Erie and found oligochaetes near the principle sources of pollution, increasing ninefold there over the number on normal areas. Tafaro (1967) collected three genera of tubificidae from southern Louisiana along the Mississippi River. Hiltunen (1967) listed twenty-six species of tubificids from Lake Michigan and noted that the presence or absence of particular species was dependant on water quality. Brinkhurst (1967) found the distribution of certain species of aquatic oligochaetes of Saginaw Bay, Lake Huron, to be influenced by the inflow of the polluted Saginaw River. Johnson and Matheson (1968) described the distribution and abundance of benthic macroinvertebrates of Hamilton Bay and adjacent Lake Ontario; Limnodrilus hoffmeisteri and Tubifex tubifex were found to be the most abundant.

Any study of the biology of the Tubificidae invariably involves some type of organic pollution associated with the organisms. Dean (1964) found that tubificids and chironomids were the only benthic forms inhabiting a chain of lakes which receives sewage regularly. Dean and Haskin (1964)

monitered the repopulation of the benthos of a river estuary following pollution abatement; the dominant forms of organisms were given and trends indicated. King and Ball (1964) devised a method of quantitatively measuring stream pollution by examining the weight ratio of aquatic insects and tubificids. Brinkhurst (1965a) summarized the knowledge concerning the tubificids, with regard to environmental tolerance limits, indicator organisms, and community structure; he also indicated future lines of profitable research. Brinkhurst and Kennedy (1965) found four species of tubificids co-existing in a polluted stream; they suggested that the food habits be investigated. King and Ball (1967) related the changes in production of a stream to the variation in its ecology as a result of periodic pollution; the relation of tubificids to the energy budget was given.

The fact that tubificids commonly inhabit water having low dissolved oxygen levels stimulates investigations into the respiratory physiology of the group. Fowler and Goodnight (1962) measured the respiration of tubificids using the Warburg apparatus by altering the environment of the worms. Whitten and Goodnight (1966a) determined the gross chemical composition of T. tubifex and L. hoffmeisteri and found that the latter had a higher percentage of lipid-soluble material probably being due to its having more chloragogen tissue. Palmer (1966) described the capillary systems of the intestine and body wall and (1968) correlated oxygen content of rivers with T. tubifex distribution.

A small amount of work has been done with the embryology of the tubificids. Weber (1962) attempted to relate the ultrastructure of ovocytes and embryonic cells to their morphogenic organization. Matsumoto (1963) studied the ash distribution in the maturation and cleavage stages of the eggs of Tubifex hatti. Inase (1967) investigated the behavior of the pole plasm in the early development of T. hatti.

Aquatic annelids often serve as intermediate hosts for the larval development of various fish parasites. The effect of temperature as a stress on the development of Biacetabulum macrocephalum (Cestoda) was investigated by Buchwald and Ulmer (1964). Tubificids may serve as an intermediate host for Archigetes sieboldi, a cestode, as described by Calentine and DeLong (1966). The larval development of Glaridacris confusa (Cestoda) occurs in tubificids as well as in other aquatic annelids (Calentine and Williams, 1967).

Tubificids are important as food in the culture of fish. Foster, Scheier, and Cairns (1966) noted the negative effect of alkyl benzene sulfonate (ABS) on the feeding of flagfish on tubificids. Ghabbour (1966) discussed the general use of aquatic oligochaetes as fish food.

The toxicity of compounds to tubificids has been investigated. Whitten and Goodnight (1966b) determined the toxicity of some common insecticides to tubificids. Coler, Gunner, and Zuckerman (1968) gave the tolerance limits of tubificids to streptomycin.

MATERIALS AND METHODS

In June, 1969, and March, 1970, mud samples containing tubificid worms were taken from along the banks of the Kickapoo Drainage Ditch. The top ten centimeters of mud was removed with a common spade, placed in plastic buckets, covered with an inch of water, and transported back to the laboratory.

The mud was spooned into sieves of the U. S. Standard Sieve Series having 1. 68 mm and 2. 00 mm openings. A slow stream of room-temperature tap water was applied and the mud washed through the sieves. The sieves were then placed in white porcelain trays with just enough water to cover the mesh. The exposed tubificids were separated from the detritus by aspiration with a large-mouth eyedropper.

The tubificids were maintained in an aerated glass aquarium containing a mixture of two liters tap water and two liters Knop's solution. The water in the aquarium was allowed to stand one day before using. The tubificids were used within one week after being placed in the aquarium.

Prior to the sterilization procedure, the entire working area was washed with 70% ethanol. Thirty-five tubificids were removed from the aquarium and placed in a holding dish. Five of these worms were then

transferred to each of seven petri dishes containing sterile Knop's solution at room temperature. Each petri dish was then moved to an ultraviolet light chamber and placed at a distance of 11.4 cm from the light source. A General Electric tubular 15-watt low pressure germicidal lamp 45.72 cm long and 2.54 cm in diameter was used. The petri dish was placed below the light, the cover removed and inverted at its side. After an exposure time of thirty seconds, the glass cover was replaced and the dish removed; each dish of tubificids was sterilized separately. The light was turned on for ten minutes before use.

Each tubificid was aseptically transected three times along its length; the cut ends only were then touched to the surface of a sterile nutrient agar bacteriological plate in a regular pattern. All thirty-five tubificids were cut and plated resulting in thirty-five culture plates. The culture plates were incubated for seventy-two hours at 37° Centigrade.

Isolated pure cultures were obtained from the resulting mixed cultures by employing the streak-plate technique: portions of the mixed cultures were streaked across nutrient agar plates with a sterile transfer loop. By successive plating of the resulting isolated colonies, pure cultures were obtained. The various cultures to be identified were selected on the basis of their differing pigmentations, size and growth rates, margins, elevations, and optical features. Only microorganisms of the class Schizomycetes were considered in this study.

The following observations were made as aids to the identification of the pure cultures:

I. The cultural characteristics of the individual pure colonies were noted:

A. Pigmentation - the color of the culture

B. Size and growth rate - the rapidity of growth while cultured

C. Margin - the condition of the periphery of the colony

D. Elevation - the degree of convexity or concavity of the colony

E. Optical features - the degree of transparency of the colony

II. The morphology of the individual cells was examined with both the phase contrast and light microscopes:

A. Type and arrangement -

1. Coccus - a spherical cell

a. Diplococcus - a pair of cocci
b. Streptococcus - a chain of cocci in one plane
c. Tetracoccus - four cocci in one plane
d. Staphylococcus - an irregular pattern of cocci in three planes
e. Sarcina - a cuboidal arrangement of cocci in three planes

2. Bacillus - a rod-shaped cell

a. Diplobacillus - a pair of bacilli
b. Streptobacilli - a chain of bacilli

3. Spirillum - a spiral-shaped cell

B. Size - the relative size of the cell

C. Endospores - the presence and location of endospores and the condition of the sporangium was noted

III. A Gram stain of each culture was made using the conventional reagents and procedure:

An air-dried bacterial smear was made on a clean slide using sterile water.

The surface was flooded with crystal violet for one minute.

The slide was rinsed in running tap water and blotted dry.

The surface was flooded with Lugol's iodine for one minute.

The slide was rinsed in running tap water and blotted dry.

The slide was destained with 95% ethanol for thirty seconds.

The slide was rinsed in running tap water and blotted dry.

The slide was counterstained with safranin for twenty seconds.

The slide was then rinsed in running tap water, blotted, and allowed to air dry before being examined.

IV. Various physiological tests were employed to further characterize the unknown cultures:

A. Carbohydrate broths were used to test the fermentation capabilities of the cultures. Phenol red, having a pH range of 6.8 to 8.4, was used as a relative pH indicator. The carbohydrates, used in 1% final concentrations, were:

arabinose
glucose (dextrose)
xylose
lactose
glucose
raffinose
mannitol

the pH was adjusted with 1N HCl or 1N NaOH so that the color of the media was a salmon pink or alkaline

The same carbohydrate broths were used with an ammonium salts base by the addition of the following:

$NH_4H_2PO_4$	1.0 g
KCl	0.2 g
$MgSO_4 \cdot 7H_2O$	0.2 g
Distilled water	1000 ml

adjust pH to 7.2 - 7.3

B. IMViC Tests - Used only on Gram negative, asporogenous bacilli which reduce nitrates to nitrites and ferment glucose with the production of acid and gas (Enterobacteriaceae).

1. Tryptone broth - The broth was prepared in accordance with the directions of the manufacturer. The presence of indole was tested for by the addition of several drops of Kovac's reagent:

75 ml amyl or isoamyl alcohol
5 g p-dimethylaminobenzaldehyde
25 ml concentrated HCl

The presence of a red layer was a positive test for the production of indole.

2. Methyl red-Voges-Proskauer Tests - The broth was prepared in accordance with the directions of the manufacturer. Two tubes of the broth were used in the test.

First tube: Several drops of methyl red indicator were added:

0.1g methyl red
300.0 ml 95% ethanol

The presence of a red color was a positive test for the production of acid; a yellow color was negative.

Second tube: Fifteen drops of alpha-naphthol were added:

5% solution of alpha-naphthol or
1-naphthol in 95% ethanol

Fifteen drops of 40% KOH were added:

40 g of KOH in 100 ml distilled water

The tube was periodically vigorously shaken over a period of fifteen minutes and allowed to stand. A red color was a positive test for the production of acetylmethylcarbinol.

3. Simmons Citrate Agar - The agar slant was streaked with the unknown culture. Growth on the surface of the agar slant and an accompanying change in its color from dark green to dark blue over a period of several days was a positive test, indicating that the bacteria was able to use sodium citrate as a sole carbon source with ammonium salts as the sole nitrogen source. No growth was a negative test. The media was prepared in accordance with the manufacturer's directions.

C. Other physiological media

1. Nitrate broth - The broth was prepared in accordance with the manufacturer's directions. Two drops of sulfanilic acid and two drops of alphanaphthylamine were added and mixed:

sulfanilic acid	alphanaphthylamine
350 ml distilled water	350 ml distilled water
150 glacial acetic acid	150 ml glacial acetic acid
4 grams sulfanilic acid	2.5 grams alphanaphthylamine

A red color indicated the presence of nitrite; if no red color appeared, either the nitrate was not reduced to form nitrite or nitrate was reduced to form nitrite then to something else. A very small quantity of zinc dust was added if there was no red color. A red color with the addition of zinc dust was a negative test; that is, the nitrate was not reduced.

2. Starch hydrolysis - The agar was made in accordance with the manufacturer's directions. The agar was inoculated with one straight streak of the unknown along the agar. After the culture had grown, the surface of the plate was flooded with iodine. A clear area adjacent to the line of growth with the remaining agar blue was a positive test for starch hydrolysis. No clear area was a negative test.

3. Catalase reaction - A small amount of the unknown culture was submerged in a droplet of hydrogen peroxide. The formation of gas bubbles was a positive test for the enzyme catalase. No bubbles formed was a negative test.

4. Salt broth - Nutrient broth was prepared in accordance with the manufacturer's directions. The appropriate amount of salt was then added to make the final desired salt concentration. The turbidity of the broth was used as the criterion for growth.

5. Litmus milk - The litmus milk was prepared in accordance with the manufacturer's directions. A red color after inoculation was evidence of acid production; blue, of alkaline conditions. The litmus may be reduced which would be evident by the medium becoming white. The milk may coagulate.

6. Gelatin liquefaction - The ability to liquify gelatin was examined by inoculating nutrient gelatin prepared according to the manufacturer's directions. The medium was incubated at 20°C. for at least five days. The obvious liquefaction of the gelatin was taken as evidence of the proteolytic ability of the culture.

7. Potato growth - The ability of a culture to grow on potato was examined by inoculating a sterile slice of raw potato. The abundance of growth and colony color was noted.

D. The motility of the cells was determined by two methods:

1. The direct observation of living cells under high power-oil immersion optics. The extended unidirectional movement of the cells in random directions was considered evidence of the motility of the culture.

2. Lennox motility medium was used:

nutrient gelatin	8.5 grams
yeast extract	0.3 grams
tryptone	2.0 grams
agar	0.4 grams
distilled water	100 ml

The sterile medium was inoculated by stabbing through its center with a straight needle. The macroscopic diffuse zone of growth from the line of inoculation was then taken as evidence of motility. The medium was examined at 12, 24, and 48 hours.

A Gram stain was made of each culture in order to ascertain the Gram reaction and the morphology of the cells; the stain was also used as a check on the purity of the culture. Certain information was acquired from each culture--colony characteristics, motility, tolerance to oxygen, catalase reaction, nitrate reduction, gelatin liquefaction, and the ability to ferment various carbohydrates.

The colonies were classified according to the one of the following four types. The appropriate physiological tests were then performed on those cultures.

Gram negative bacillus:	litmus milk growth on potato indole production Voges-Proskauer-Methyl Red reactions growth on Simmon's citrate
Gram positive bacillus:	endospore check acetonin production starch hydrolysis growth in 7% salt media
Gram negative coccus:	none
Gram positive coccus:	litmus milk growth on potato growth in 7% salt media

The results of the tests were compiled and considered in determining the genus and species of the pure culture, using the Difco Manual (1953) and Bergey's Manual of Determinative Bacteriology (Breed, 1957).

RESULTS

The following pure cultures were isolated and identified:

Division Protophyta

Class Schizomycetes

Order Eubacteriales

Rigid bacilli or cocci; occur singly or in chains; not acid fast; reproduction by transverse fission; pigments of chromogenic species are commonly non-water soluble and of a carotenoid nature; saprophytes, parasites, and pathogens; found in fresh and salt water, air, soil, and in bodies of plants and animals.

Family Achromobacteraceae

Small to medium-sized Gram-negative bacilli; many yellow chromogens; primarily saprophytes in foods, soil, and fresh and salt waters; less commonly as parasites and pathogens.

Genus Achromobacter

No pigment formed; litmus milk faintly acid to unchanged or alkaline; occur in salt- to fresh-water and in soil.

Achromobacter eurydice - aerobic, facultative; acid from glucose, but little or no action from other carbohydrates; of nineteen amino acids tested, none was required for growth; ammonium chloride and the nineteen amino acids may serve as sources of nitrogen; trimethylene not produced from oxide, betaine, choline, or acetyl choline; inorganic sulfur may serve as a source of sulfur; innocuous when fed to bees; not pathogenic when inoculated subcutaneously in rabbits; occurs as a secondary invader on European foulbrood of bees; habitat: unknown.

Achromobacter parvulus - strict aerobe; optimum temperature 25°C; nitrates reduced; grows poorly in liquid media; no acid from glucose, lactose, sucrose, glycerol, or ethanol in either liquid or solid media; nitrates reduced; causes strong volatilization of ammonia from a mixture of horse manure and urine; habitat: soil.

Achromobacter pestifer - aerobic, facultative; optimum temperature 25°C; growth range 10° - 30°C; nitrates reduced; no action on carbohydrates; ammonia not produced from peptone; urase not produced; trimethylamine oxide not reduced; non-hemolytic; not lethal to white mice when injected in massive doses; does not cause soft rot on carrots potatoes, or turnips; habitat: presumably widely distributed in water, soil, and foodstuffs.

Genus Alcaligenes

Litmus milk alkaline with or without peptonization; carbohydrates not utilized; acetonin not produced; generally found in intestinal tracts of vertebrates or in dairy starters; indole not produced.

Alcaligenes faecalis -aerobic; optimum temperature 25° - 37°C; nitrate may be reduced; aliphatic amides toxic; aspartic acid, asparagine, histidine, and glutathione support good continued growth; non-pathogenic; habitat: widely distributed in decomposing organic matter.

Alcaligenes marshallii - aerobic; optimal temperature 30°C; nitrates not reduced; habitat: milk.

Alcaligenes metacaligenes - aerobic, facultative; optimum temperature 22°C; nitrate reduction variable;habitat: intestinal canal.

Genus Flavobacterium

Commonly proteolytic; fermentive metabolism usually not conspicuous; acid reactions commonly do not develop from carbohydrates when available nitrogen-containing organic compounds are in the medium; no gas from carbohydrates; simple nutritional requirements; in water and soil; some pathogenic.

Flavobacterium arborescens - aerobic, facultatively anaerobic; optimum temperature 30°C; no growth in nitrate solutions; habitat: water.

Flavobacterium breve - aerobic, facultatively anaerobic; optimum temperature 35°C; gelatin not liquefied; pathogenic for laboratory animals; habitat: sewage.

Flavobacterium lutescens - aerobic, facultatively anaerobic; optimum temperature 30° to 35°C; nitrates reduced; habitat: fresh and salt water.

Family Micrococcaceae

Gram-positive cocci; occur singly, pairs, tetrads, packets, irregular masses, or chains; heterotrophic; aerobic species do not produce gas from carbohydrates; aerobic to strict anaerobic; free-living, saprophytic to parasitic or pathogenic; aerobic forms found on skin, in skin glands or their secretions of the Vertebrata.

Genus Gaffkya

Occur in animal body as tetrads, in ordinary media as pairs and irregular masses; parasitic.

Gaffkya tetragena - aerobic, facultatively anaerobic; optimum temperature 37°C; indole not produced; gelatin not liquefied; hydrogen sulfide not produced; acid from glucose, lactose, and glycerol; starch not hydrolyzed; nitrates not reduced; ammonium salts not utilized; biotin, L-tyrosine, and L-glutamate required for growth; pathogenic for mice and guinea pigs; rabbits less susceptible; habitat: mucous membrane of the respiratory tract.

Genus Micrococcus

Occur in irregular masses, never in packets; non-chromogenic to yellow, orange, or red; catalase positive; saprophytic, facultatively parasitic, or parasitic; never truly pathogenic.

Micrococcus candidus - aerobic; optimum temperature 25°C; nitrates not reduced; acid from glucose, sucdore, lactose, and glycerol; ammonia produced from peptone; ammonium salts not utilized; non-pathogenic; habitat: skin secretions, milk and dairy products.

Genus Sarcina

Division in three perpendicular planes producing regular packets; aerobic and anaerobic; saprophytic and facultatively parasitic.

Sarcina flava - aerobic; optimum temperature 30° to 35°C; gelatin slowly liquefied; indole not produced; nitrates not reduced; habitat: air, water, soil.

Family Neisseriaceae

Gram-negative cocci; occur in pairs or masses; aerobic to anaerobic; all parasitic.

Genus Neisseria

Cocci nearly 1.0 micron in diameter; occur in pairs with adjacent sides flattened; indole not produced; nitrates not reduced; catalase abundantly produced; animal parasites.

Neisseria catarrhalis - aerobic, facultatively anaerobic; optimum temperature 37°C; grows well at 22°C; no acid from any carbohydrate; habitat: human mucous membrane of the respiratory tract; may be associated with other animals having inflamations of the mucous membrane.

Neisseria caviae - aerobic, facultatively anaerobic; optimum temperature 37°C; grows at 22°C; no acid from any carbohydrate; some strains weakly hemolytic against rabbit blood; habitat: pharyngeal region of guinea pig and perhaps from pharyngeal region of other animals.

Family Brevibacteriaceae

Bacilli, ranging from very short, almost coccoid forms to rather long bacilli; Gram-positive; red to yellow to orange to brown pigments may be produced; aerobic to facultatively anaerobic; occur in dairy products, soil, salt- and fresh-water and decomposing substances.

Genus Brevibacterium

Short bacilli; sometimes chromogenic with non-water-soluble pigments; aerobic to facultatively anaerobic; rarely microaerophilic; found in dairy products, soil, salt- and fresh-water and decomposing matter.

Brevibacterium ammoniagenes - aerobic, facultatively anaerobic; optimum temperature 30°C; nitrates reduced; no action on carbohydrates; urea fermented forming ammonia; blood serum not liquefied; optimum pH 7.0 to 8.5; not pathogenic for rabbits or guinea pigs; cause of diaper rash of infants; habitat: presumably widely distributed in putrefying materials.

Family Corynebacteriaceae

Bacilli; generally Gram-positive; wedge and club forms common; animal and plant parasites and pathogens.

Genus Arthrobacter

Young cultures have irregularly-shaped bacilli being bent, angular, club-shaped, swollen; coccoid cells develop in older cultures; little or no acid from carbohydrates; indole not produced; little or no growth at 37°C; typically soil organisms.

Arthrobacter aurescens - aerobic, facultatively anaerobic; optimum temperature 20° to 32°C; slight growth at 10°C and 37°C; no growth at 45°C; nitrates reduced; hydrogen sulfide produced in cysteine and thiosulfate media; slight acidity from glucose and sucrose; no gas from carbohydrates; starch hydrolyzed; uses nitrates and ammonium salts as nitrogen sources; citrates used as sole carbon source; habitat: soil.

Arthrobacter citreus - aerobic; optimum temperature 25° to 30°C; grows well between 20° to 32°C; fair growth at 10°C; little or no growth at 37°C; habitat: soil.

Family Bacillaceae

Bacilli capable of producing endospores; generally Gram-positive; sporangia may be swollen; gelatin frequently hydrolyzed; sugars generally fermented sometimes with production of gas; aerobic to anaerobic; saprophytic in soil; some are animal or insect parasites or pathogens.

Genus Bacillus

Proteins generally decomposed with the production of ammonia; catalase positive; mostly saprophytes; commonly in soil; some are insect parasites or pathogens.

Bacillus cereus mycoides - aerobic to facultative anaerobic; optimum temperature 30°C; maximum 37° to 48°C; gelatin liquefied; acetonin produced; citrates used as sole carbon source; amino acids necessary for growth; lecithinase produced; habitat: widely distributed in soil, dust, milk, and plant surfaces.

TABLE I. Summary of Species Isolated and Identified

Division Protophyta

Class Schizomycetes

Order Eubacteriales

I. Family Achromobacteraceae

A. Achromobacter eurydice
B. Achromobacter parvulus
C. Achromobacter pestifer

D. Alcaligenes faecalis
E. Alcaligenes marshallii
F. Alcaligenes metalcaligenes

G. Flavobacterium arborescens
H. Flavobacterium breve
I. Flavobacterium lutescens

II. Family Micrococcaceae

J. Gaffkya tetragena

K. Micrococcus candidus

L. Sarcina flava

III. Family Neisseriaceae

M. Neisseria catarrhalis
N. Neisseria caviae

IV. Family Brevibacteriaceae

O. Brevibacterium ammoniagenes

V. Family Corynebacteriaceae

P. Arthrobacter aurescens
Q. Arthrobacter citreus

VI. Family Bacillaceae

R. Bacillus cereus mycoides

TABLE II. A Summarization of the Physiological Characteristics of Bacteria Isolated From the Gut of Tubificid Worms

Culture	Morphology	Colony	Oxygen Requirements	Gram Reaction	Motility	Spores	Gelatin Liquefaction	Catalase	Diastase	Nitrates Reduced	Indole Produced	Acetonin Produced	Methyl Red	Sodium Citrate Used	Litmus Milk	Potato Growth	Nutrient Broth
A	bacillus	white/gray	aerobe, facultative	-	-	-	-	+	-		-	-			NC	slight, gray	turbid, sediment
B	bacillus	gray	strict aerobe	-	-	-	-	+	-	+	-	-			NC		turbid
C	bacillus	gray	aerobe, facultative	-	+	-	-	+	-	+	-	-	-	-	NC	gray	turbid, thin pellicle
D	bacillus	white/gray	aerobe	-	+	-	-	+	-	-	-	-			alk.	Moderate, brown/yellow	turbid, thin pellicle
E	bacillus	gray then yellow	aerobe, facultative	-	-	-	+	+	-	-	-	-			alk. pep. odor	heavy, yellow	turbid, ring, sediment
F	bacillus	gray	aerobe, facultative	-	-	-	-	+	-	+	-	-			alk.	scant	pellicle, sediment
G	bacillus	dark orange	aerobe, facultative anaerobe	-	-	-	+			unk	-	-			neut. Reduced coag.	heavy dark orange	turbid, sediment

(TABLE II. - continuation)

Culture	Morphology	Colony	Oxygen Requirements	Gram Reaction	Motility	Spores	Gelatin Liquefaction	Catalase	Diastase	Nitrates Reduced	Indole Produced	Acetoin Produced	Methyl Red	Sodium Citrate Used	Litmus milk	Potato Growth	Nutrient Broth
H	bacillus	Pale yellow	aerobe, facultative anaerobe	-	-	-	-				-	-				NG	turbid
I	bacillus	Yellow	aerobe, facultative anaerobe	-	-	-	+			+	-				alk.	heavy, yellow	turbid
J	coccus, tetrads	white	aerobe, facultative anaerobe	+	-	-	-	+	-	-	-	-			slight acid	white	clear sediment
K	coccus	white	aerobe	+	-	-	-	+	-	-	-	-			slight acid	heavy, white	turbid, pellicle
L	coccus, packets	yellow	aerobe	+	-	-	+	+	-	-	-	-			alk. no coag.	yellow	slightly turbid, sediment
M	coccus, pairs, flat sides	white/gray	aerobe, facultative anaerobe	-	-	-		+		-	-	-			NC		turbid, slight pellicle
N	coccus, pairs, flat sides	tan	aerobe, facultative anaerobe	-	-	-		+		-	-	-			NC		turbid
O	bacillus	gray	aerobe, facultative anaerobe	+	-	-	-	+		+	-	-			slight alk.		turbid
P	irregular bacillus & coccus	yellow	aerobe, facultative anaerobe	Var.	-	+	+	+	+	+	-	-		+		brown/ yellow	turbid, sediment
Q	V-shaped bacillus, irregular	yellow	aerobe	Var.	Var.	+	+	+	-	+	-	-		-		scant yellow	turbid, sediment
R	bacillus	gray, sinistral whorls	aerobe, facultative anaerobe	+	-	+	+	+	+	+	-	+		+	pep.	heavy white	turbid, sediment

TABLE III. A Summarization of Carbohydrate Utilization by Bacteria Isolated From the Gut of Tubificid Worms

Carbohydrate	Culture																	
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R
Arabinose	-	-	-	-	-	-							-	-	-	-	-	-
Xylose	-	-	-	-	-	-							-	-	-	-	-	-
Glucose	+	-	-	-	-	-	-	-	-	+	+	+	-	-	-	+	-	+
Lactose	-	-	-	-	-	-	-	-	-	+	+		-	-	-	-	-	-
Sucrose	-	-	-	-	-	-		-	-		+		-	-	-	+	-	+
Raffinose	-	-	-	-	-	-							-	-	-	-	-	
Mannitol	-	-	-	-	-	-			-				-	-	-	-	-	-
Glycerol	-	-	-	-	-	-				+	+		-	-	-	-	-	+
Dulcitol	-	-	-	-	-	-							-	-	-	-	-	

DISCUSSION

Fresh sewage is an aquaeous mixture of chemicals and solids in suspension and solution with an assortment of floating articles; the concentration of the sewage is directly related to the amount of water present. Sewage water contains various organics: nitrogenous compounds, such as urea, proteins, amines, and amino acids; and non-nitrogenous compounds, such as fats, soaps, and carbohydrates, including cellulose. The principle objective of the treatment process is to stabilize the organics; that is, they must be broken down by bacterial action to simple substances that cannot be further decomposed. Anaerobic bacteria are active in sludge decomposition process, while aerobic forms decompose the more aqueous sewage. The processed sewage is then disposed of by dumping into a body of water and diluted (Steel, 1960). Of the three zones of self-purification in a polluted stream--zone of immediate pollution, zone of active decomposition, and zone of recovery--the last would be descriptive of the portion of the Kickapoo where the tubificids were gathered. The sludge was less in amount and the water clearer than upstream. The levels of dissolved carbon dioxide and ammonia decrease, while that of dissolved oxygen, nitrates, and nitrites

increase. Most of the bacteria present are of the aerobic type. Tubificide act as scavengers and feed on the debris containing the bacteria.

Knowledge in the field of bacterial ecology is greatly deficient as compared to that of bacterial physiology and biochemistry (Hungate, 1962). This is due, in a large part, to the lack of economically profitable applications of such knowledge and the many uncontrollable variables in the microbial ecosystem. It is known, however, that the type of food present in the ecosystem is of primary importance in influencing the type of community which may develop.

Sterilization of the external surface of the tubificids was accomplished by the use of ultraviolet light. The lamp used was a General Electric 15-watt low-pressure germicidal lamp, emitting 90% of its energy as 2537A radiation. The lamp differed from a common fluorescent lamp only by transmitting ultraviolet light and not having the inner surface of the glass tube coated with fluorescent material. The lamp emits 2840 equivalent milliwatts at 2600A; at one meter distance, 27-31 microwatts per square centimeter of 2537A radiation is received.

It is safe to assume that nearly all of the transmitted energy reached the surface of the worm, very little being absorbed by the thin layer of Knop's solution over the surface of the worms. The absorption of ultraviolet light by distilled water in thin layers is negligible. Even at three

inches deep, 92% of 2537A energy is transmitted. The presence of dissolved salts or organic matter increases the absorption coefficient: twenty mm of sea water transmits nearly 70% of 2537A light. However, as one can see from the ingredients of Knop's solution as compared to the ingredients of an artificial solution very similar to sea water, the Knop's has far fewer dissolved salts--the absorption of the overlying water solution would be slight.

Artificial Sea Water

NaCl	23.991 g		
KCl	0.742 g		
$CaCl_2$	1.135 g	or	
		$CaCl_2 \cdot 6H_2O$	2.240 g
$MgCl_2$	5.102 g	or	
		$MgCl_2 \cdot 6H_2O$	10.893 g
Na_2SO_4	4.012 g	or	
		$Na_2SO_4 \cdot 10H_2O$	9.1 g
$NaHCO_3$	0.197 g		
NaBr	0.085 g	or	
		$NaBr \cdot 2H_2O$	0.115 g
$SrCl_2$	0.011 g	or	
		$SrCl_2 \cdot 6H_2O$	0.018 g
H_3BO_3	0.027 g		

add distilled water to one liter

Knop's Solution

$Ca(NO_3)_2 \cdot 4H_2O$	0.8 g
KNO_3	0.2 g
KH_2PO_4	0.2 g
$MgSO_4 \cdot 7H_2O$	0.2 g
$FePO_4$	trace

one liter distilled water

Far ultraviolet light, between 1800A-2900A, penetrates human skin to a depth of 0.01 to 0.1 mm. By interpolation, a wavelength of 2537A should penetrate to a depth of 0.07 mm. If one can assume the depth of penetration into the body wall of tubificids, under the conditions

of this sterilization, to be no greater, it would then be safe to conclude that the ultraviolet did not penetrate the worm sufficiently to reach the gut.

An investigation was carried out to determine the length of time necessary to expose the worms to the light for sterilization without harming the worm nor disturbing the gut flora. Worms exposed to the ultraviolet light for 1 1/2 minutes died shortly after their removal; only eight out of thirty-five plates inoculated with material from their guts showed signs of growth. Worms exposed for one minute were streaked across a sterile plate, then bisected and the gut contents plated. The Knop's solution was also streaked on a plate. Neither of the plates inoculated with the worms or the Knop's showed signs of growth. Of the five plates inoculated with gut contents, one plate had sixty colonies with the remaining plates having thirty, twenty-five, ten, and three colonies respectively.

The germicidal effectiveness of ultraviolet light is at its peak with a wavelength of 2600A. The 2537A light emitted by the nearly monochromatic light source used is close enough to the 2600A that it is a very effective bactericidal agent. Bacteria subjected to ultraviolet light do not all die at once, but a constant fraction of the population dies per unit time. That surviving fraction is termed the survival ratio. The fraction

killed is one minus the survival ratio, expressed in percentage. The survival ratio may be shown as:

$$N/N_o = e^{-KIt}$$

where N_o is the number of organisms initially present
N is the number of organisms surviving at time t
t is the time of exposure to the bactericidal agent
K is a constant which depends on the organism involved and the wavelength
I is the intensity of the ultraviolet light expressed in microwatts per square centimeter.

Temperature has very little effect on the ability of ultraviolet light to kill or render inactive bacteria (Koller, 1952).

The majority of bacterial species identified are normal inhabitants of decomposing matter, water, soil, or are associated with animals. The bacteria may have been present in the processed sewage as it left the disposal plant or may have been subsequently added to the water by being washed into the stream with any runoff water.

The fact that all cultures isolated were aerobic or facultatively anaerobic is not unusual. In the section of the drainage ditch used, the dissolved oxygen level would not be great but would be sufficient to support the aerobic growth of bacteria. That portion of the ditch farther upstream would tend to be more anaerobic. Also, no anaerobic techniques were used to isolate strict anaerobic cultures, due to lack of facilities and time. Such research is planned for the immediate future.

Brinkhurst (1970) found that some labelled bacteria when fed to tubificids were broken down and the resulting amino acids were incorporated into the chlorogogen cells of the worm. The remaining bacteria were not broken down but were maintained in the gut. Burrows (1959) stated that Gram positive bacteria are more resistant to proteolytic enzymes and the action of alkali, while Gram negative bacteria are more susceptible. Eleven of the eighteen cultures isolated in this research were Gram negative. The cell wall of bacteria, which makes up nearly 20% of the dry weight of the cell, is composed of three compounds: lipids, peptides, and polysaccharides, which are present in different proportions and amounts in different species. The cell walls of Gram negative bacteria contain much more, up to ten times more, lipid material than do Gram positive species. Up to 20% of the cell wall of Gram negative bacteria may be lipid material (Pelczar and Reid, 1965). Halvorson (1962) stated that the lipid content of Gram positive species is generally very small. Van Gansen (1956) and Roots (1960) have both found that the chlorogogen tissue of several earthworms contains phospholipids and lipid-soluble materials. Whitten and Goodnight (1966a) reported that the chlorogogen cells of both Limnodrilus hoffmeisteri and Tubifex tubifex retained Sudan Black B very strongly. This seems to indicate that at least a portion of the chlorogogen tissue is composed of lipid material. It may be that the Gram negative bacteria are being broken down

by the digestive enzymes of the tubificid gut and the components transported to the chlorogogen cells, while the Gram positive bacteria are maintained in the gut, possibly to be used in a symbiotic relationship or to be eliminated from the body.

The Gram positive bacteria found were normally inhabitants of putrefying materials, widely distributed in nature, or found associated with vertebrates. The fact that they were found in the tubificid gut was not unusual because of their being so widely distributed. Due to the lack of any other unifying characteristics of the group, it is thought that they played a small role in the nutritional physiology of the worms.

A characterization of the proteolytic enzymes of the tubificid gut needs to be investigated in order that the effect of the enzymes on the bacterial cell wall might be known, since the cell wall is sensitive only to certain proteolytic enzymes. Extensive qualitiative and quantitative investigations on the bacterial flora of the gut must be undertaken. All of the bacteria of the gut, both aerobic and anaerobic species, must be characterized, as well as their relative abundance. In this manner, the importance of different bacterial species on the nutritional physiology of tubificids may be ascertained.

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