AXENIC CULTURE AND UPTAKE OF DISSOLVED ORGANIC SUBSTANCES BY THE MARINE NEMATODE, *RHABDITIS MARINA* BASTIAN (1).

by

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Résumé

Elevage axénique et consommation de substances organiques dissoutes par le Nématode *Rhabditis marina* Bastian.

Des *Rhabditis marina* monoxéniques ont été débarrassés de leurs bactéries associées *(Pseudomonas* sp.) par lavage aseptique puis transférés dans des milieux contenant des antibiotiques. *R. marina* est maintenu en élevage axénique sur le milieu de Grace utilisé pour les Insectes, à base de sel marin. L'addition de casamino-acides, d'hémoglobine et de matériaux naturels bruts, riches en lipides, ont un effet stimulant sur la croissance et la reproduction. Des matériaux riches en lipides, tels que le TEM (triéthylèneamine) et le TWEEN 80 sont d'importants stimulants et remplacent le mélange d'amino-acides lorsqu'ils sont fournis avec de la poudre d'oeufs entiers. Des mélanges de différents acides gras peuvent remplacer des mélanges de lipides non définis. Il est probable que *R. marina* a moins de possibilités de synthèse des acides gras que les autres Nématodes libres précédemment étudiés.

La prise de plusieurs substances organiques dissoutes par *R. marina* a été étudiée. Aucune prise de substances organiques dissoutes marquées au ^{14}C n'a été observée en solution, même à de hautes concentrations (20 raM). Cependant, l'addition au milieu expérimental de petites sphères de latex (0,5-10µ) a permis la prise d'éléments marqués. Cela suggère que le principal passage de nourriture se fait probablement, pour *R. marina,* à l'aide de matériel particulaire tel que les Bactéries et, peut-être, les Algues.

Introduction

Despite the widespread occurrence and abundance of free-living marine nematodes successful continuous culture of these organisms in the laboratory has been limited to but a few species (see Tietjen and Lee, in press, for a summary). Most of these cultures have been agnotobiotic; however, there are four species, *Rhabditis marina, Monhystera denticulata, Chromadora macrolaimoides* and *Chromadorina germanica* which have been grown in gnotobiotic culture (Tietjen et

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al., 1970; Tietjen and Lee, 1972, 1973; Tietjen and Lee, in press). All of these cultures have either been monoxenic, dixenic or trixenic; to our knowledge, there have been no published reports of marine nematodes in axenic culture.

This paper will be concerned with the methods by which one nematode, *Rhabditis marina* Bastian, was established in axenic culture; information on some of its nutritional requirements and uptake of dissolved organic substances will also be presented and discussed.

Axenic culture is necessary to properly study the nutrition and uptake of dissolved organic substances by any organism. It is only in this way that the activities of the organism under study can be separated from co-existing organisms. Among the invertebrates, nematodes have attracted the attention of many workers in the field of axenic cultivation. An excellent review of the culture methods used in nematology (both for free-living and parasitic forms) was published by Rothstein and Nicholas (1969) ; to this should be added the reports of Gerlach and Schrage (1971, 1972), Hopper et al. (1973), and the reports of work from our own laboratory (Tietjen et al., 1970; Tietjen and Lee, 1972, 1972; Tietjen and Lee, in press).

AXENIC CULTURE OF *RHABDITIS MARINA* AND NUTRITIONAL STUDIES

Rhabditis marina has been established in reliable monoxenic culture on a single bacterium, *Pseudomonas* sp. (Tietjen et al., 1970). These well established monoxenic cultures were used as stocks for axenic isolation. Although the organisms are quite euryhaline and eurythermal (Tietjen et al., 1970), they are easily damaged by frequent manipulation during experimental procedures and thus must be handled quite gently. The first step in axenic culture involved the reduction and eventual elimination of the associated *Pseudomonas.* Two washing procedures were employed: (1) gentle (by micropipette) aseptic transfer of individual nematodes through sterile sea water in the wells of 9-hole Pyrex spot plates and (2), gentle agitation of the nematode cultures in sterile screw-cap test tubes containing concentrated antibiotic solutions, followed by slow centrifugation in a hand centrifuge. This procedure was followed by withdrawal of the overlying sea water and antibiotics and replacement with sterile sea water. After 20-30 washes by either method, bacterial counts on sterility test media were reduced to very low levels (less than one cell/ml). Phase contrast observation of the supernatent fluid and of the nematodes themselves failed to reveal the presence of any bacteria.

In order to isolate the nematodes into axenic culture, three serial transfers in media containing Erythromycin (200-330 μ.g/ml) were necessary. Initially, the viability of newly isolated worms was very low: contributing factors were experimental manipulation, antibiotic toxicity (also shown for *Chromadora macrolaimoides* by Tietjen and Lee, 1973) and an inadequate substitute diet. On the first media tried as a substitute for living bacteria, axenic cultures could not be main-

tained for more than three generations. It was necessary, therefore, to continually reisolate new nematodes. We found that viability of the cultures could be enhanced without loss of antibiotic effectiveness by lowering the concentration of Erythromycin to 100 ug/ml, supplemented with Chloramphenicol (2 ug/ml) and Polymyxin-B (0.6 ug/ml).

In the earliest stages of isolation, growth was best in marine nutrient agar (a bacteriological medium made with 30 mg nutrient agar per ml sea water, diluted to 50 percent normal concentration with distilled water) and Medium 10 (see Lee et al., 1970, for composition of Medium 10) plus 0.05 percent (w/v) Difco Bactopeptone. Various peptones, crude natural materials and mixtures of metabolites which have often proven to be good nutritional supplements for other organisms, including free-living soil nematodes (Hutner et al., 1957; Holtz, 1964; Lee et al., 1970; Rothstein and Nicholas, 1969; Sayre et al., 1963; Tomlinson and Rothstein, 1962) would not substitute for living bacteria. Sonicated bacteria were satisfactory substitutes but autoclaved bacteria were not. Stocks of sterile filtrates of sonicated bacteria remained active for several weeks when stored in a freezer at — 18 C.

In the early 1950's, Dougherty (1950, 1951) postulated that an unidentified growth factor, termed factor R_b, was essential for the axenic culture of *Caenorhabditis briggsae,* a species closely related to *R. marina.* He further suggested that this growth factor is associated with protein and indicated that activity of this factor was very high in beef and horse liver.

We chose liver strip media for maintaining our first axenic stocks of *R. marina.* Thin strips of fresh beef liver, carefully autoclaved (5 minutes at 15 lbs psi) were found to contain the nutrients necessary for the replacement of live bacteria. Activities of the factor(s) in the liver diminished, however, in unrefrigerated storage of the liver strip media. Liver media stored under refrigeration displayed high activity, although it was necessary to pre-incubate it several days before use as a sterility check due to the fact that bacterial spores withstood the brief autoclaving. Our initial axenic culture medium consisted, therefore, of marine nutrient agar slants in screw-cap test tubes, incubated on their sides with thin strips of fresh beef liver added. Incubation temperature was 25 °C.

Initial nutritional experiments were directed toward testing media and growth factors which support the continuous growth and reproduction of the free-living and parasitic nematodes which have been grown in axenic culture *{Caenorhabditis briggsae, C. elegans, Rhabditis anomala, Turbatrix aceti, Panagrellus redivivus, Acrobeloides buetschli, Pelodera strongyloides, Neoaplectana glaseri* and *N. carpocapsae).* All of the above species and *R. marina* belong to the order Rhabditida. Culture methods for these species are summarized in Rothstein and Nicholas (1969). Among the many media tested were modified versions of the *C. briggsae* maintainance medium of Hansen (see Nicholas, 1962) ; Medium M 199, another prepared organic medium (both from the Grand Island Biological Company, Grand Island, New York), and Grace's (1962) insect medium, a highly enriched nutritive medium containing vitamins, sugars, fatty acids and amino acids (GIBCO, Grand Island, N.Y.). To grow *R. marina,* we found it necessary to replace the basic salt concentrations in the media with

a marine salt mixture (Table 1). To all three media nutrient supplements such as yeast extract, trypticase, malt extract, whole sheep blood and casamino acids were aseptically added.

Growth and reproduction were consistently better in the modified (substituted salts without insect hemolymph) Grace's medium than in the modified C. *briggsae* medium or the M 199. Addition of 10 percent whole sheep blood and casamino acids (21.662 g/L) stimulated growth and reproduction. Aseptically-added filter-sterilized microbiological grade bovine hemoglobin (1000 mg/L) was a satisfactory substitute for whole blood; neither autoclaved whole blood nor auto-

TABLE 1

Development of the culture medium for *Rhabditis marina*

	mg/L
1. Salts (Base)	
NaCl	25,000
$MgSO_4 . 7H_2O$	9,000
KCl	700
Ca (as Cl^-)	300
$Na\ SiO_3 . 9H_2O$	70
$NaHCO_3$	100
$NaH_2PO_4 . H^2O$	10
NTA	70
Tris	1.000
P II Metals	30 ml
2. Amino Acid Component I	
L-Arginine hydrochloride	1,400
L-Histidine (free base)	400
L-Lysine monohydrochloride	1,400
DL-Tryptophan	400
DL-Phenylalanine	1,000
3. Amino Acid Component II	
DL-Methionine	660
DL-Serine	1,000
L-Threonine	1,200
L-Leucine	2,400
L-Isoleucine	800
DL-Valine	1,000
4. Amino Acid Component III	
DL-Glutamic Acid (monohydrate)	3,000
L-Aspartic Acid	1,200
DL-Alanine	1,000
L-Proline	800
L.-Hydroxyproline	200
Glycine	1,000
L-Glutamine	2,000
5. Amino Acid Component IV	
L-Cysteine	2
L-Tyrosine	800
6. PPN Mix	
Xanthine	3
Uracil	3
Thymine	3
Guanine	3
Adenine	100
Hypoxanthine	3
ATP	100
D-2 Desoxyribose	5
Adenosine 3'-(2') phosphoric acid	2
7. Vitamin Mix 8 B	10 ml/L
8. Hemoglobin	100
9. Fatty Acid Component	
Oleic	1-10
Palmitic	1-10
Linolenic	1-10
Linoleic	1-10
Stearic	1-10

claved hemoglobin, however, were stimulatory. Substitution of the hemoglobin with either heme, hematin or ferratin proved to be unsuccessful.

The growth factor(s) present in the casamino acid mixture were also found in trypticase (500 mg/L) and yeast extract (500 mg/L), although reproduction occurred faster in the vitamin-free casamino acid mixture (generation time, 6 days) than in the trypticase and yeast extract (generation time, 11 days). At the above concentrations of trypticase and yeast extract, it was possible to stimulate growth even further with the addition of 100 mg/L of hemoglobin.

As a result of the success with Grace's medium, it became a base for further experiments. The animals were maintained in Grace's medium by adding pre-sterilized medium to the solidified salt base

and slanting the tubes on their sides (Tietjen et al., 1970). The nematodes were transferred once a week with the weekly addition of antibiotics and thus maintained for more than 75 generations.

Having seen that certain amino acids, vitamins and hemoglobin were at least stimulatory in certain minimum concentrations, attention was focused on lipids. The addition of various peptones, carbo hydrates or yeast extract to Grace's medium did not stimulate further growth; materials rich in lipids, however (egg yolk, whole egg, lact-albumin), did. Both hexane and aqueous extracts of whole egg powder were stimulatory at low concentrations (10-25 mg/L) when added to the Grace's medium. In the same concentration range, some growth stimulation was observed in media enriched with cephalin, egg lecithin and animal lecithin. Soy lecithin was initially stimulatory, but in the absence of other factors, growth quickly diminished. Beef tallow extracts, such as TEM (triethyleneamine, 10 mg/L) and TWEEN-80 (5 mg/L) were consistently stimulatory, and replaced the casamino acid mixture in Grace's medium when supplemented with whole egg powder. Mixtures of oleic, palmitic, linolenic, linoleic and stearic acids in low concentrations (1-10 mg/L) replaced the undefined lipid mixtures. We were unable to replace the "egg yolk factor" with cholesterol and/or mixtures of other sterols (ergosterol, beta sitosterol) for more than but a few generations in the range of concentrations used (1-10 mg/L).

Experiments aimed at developing a minimum defined medium were in progress when we moved into a new laboratory building. Incubator malfunctions destroyed our axenic stocks before we completed the study; the development of the medium up to the time of this writing is given in Table 1.

Summary of *Rhabditis marina* nutrition

Although less than a score of nematodes have been studied in axenic culture, a great deal is known about the nutrition and metabolic pathways of large numbers of representative types because large numbers (10^4-10^8 or more) can be cultured in fairly routinely prepared media. The free-living nematodes in culture are somewhat unusual animals in that they have glyoxalate cycle enzymes and abilities for *de novo* synthesis of polyunsaturated fatty acids (Rothstein, 1970; Rothstein and Gotz, 1968). They also have absolute requirements for sterols (50 mg/L) (Hieb and Rothstein, 1968) and heme (500 mg/L) (Hieb et al., 1970). Myoglobin, hemoglobin and cytochrome c can be active as replacements for the heme requirement (Rothstein, 1974). In experiments with *C. briggsae,* cytochrome c is as good as myoglobin in meeting growth requirements; in *Turbatrix aceti,* however, it is not. This suggested to Rothstein (1974) that the heme moiety is not all that is required. Growth in *C. briggsae* is also stimulated by factors present in liver (Sayre et al., 1963), chick embryo extract (Dougherty et al., 1959), yeast extract (Lower and Buecher, 1970) and yeast ribosomes (Van Fleteren, 1973). Rothstein (1974) suggests that a small peptide is the active soy peptone growth factor for C. *briggsae.* It has yet to be characterized, although Van Fleteren

(1974) has recently found evidence that the growth factor may serve as a complexing vehicle to provide hemin in a particulate form since lecithin-hemin particles were very active in stimulating the growth and reproduction of *C. briggsae.*

Although the growth and reproduction of *Rhabditis marina* was greatly stimulated by hemoglobin, we did not find it to be an absolute requirement. In this respect, *R. marina* differs from the other species of free-living nematodes whose nutrition has received attention. Furthermore, we were unable to replace the "egg yolk factor" for *R. marina* with cholesterol or mixtures of sterols (ergosterol, beta sitosterol) ; we did, however, find activity in cephalin, various lecithins, and beef tallow preparations (TEM, TWEEN 80). We suspect that this worm has fewer fatty acid synthetic abilities than the other axenic nematodes which have been studied. In the ecological sense. the points are really mute, since *Rhabditis marina* probably has rather complex nutritional requirements which restrict its growth and reproduction in the absence of food organisms which contain sufficient quantities of the necessary growth factors. The organism has been shown to be somewhat selective in its feeding activities (Tietjen et al., 1970; Tietjen and Lee, in press).

UPTAKE OF DISSOLVED ORGANIC SUBSTANCES

Once *R. marina* was established in axenic culture, experiments aimed at examining the uptake of dissolved organic substances were begun. Axenically cultured worms were aseptically transferred to the wells of 9-hole spot plates containing the marine salt mixture given in Table 1. ^{14}C-labelled, 1 uM concentrations of the following organic molecules were added: D-glucose, maltose, sucrose, glycine, L-serine, aspartic acid and acetic acid. The compounds were diluted with unlabelled substrates to give specific activities of 0.5 or 1.0 uCi/uM. Ten to thirty worms were used for each experiment, with dead worms serving as controls. The nematodes were allowed to stand overnight. They were then washed once more and placed in scintillation vials for counting.

At 1 uM levels there was no uptake of label. Progressively, higher concentrations of substrate were tested until concentrations which were environmentally improbable (20 mM) were reached; there continued to be no uptake of label.

In the next series of experiments, ^{14}C-labelled glycine, aspartic acid, glucose, sucrose, acetate and sodium bicarbonate were added at 20 mM concentrations to the spot plates but in addition, sterile latex microspheres (Minnesota Mining and Manufacturing Co., St. Paul, Minnesota) of different size classes (0.5-20 u) were also added. At 15 u, there was no significant uptake of label above background. However, with microspheres 10 u or less in diameter, there was a significant increase in the number of microspheres ingested and

uptake of label (Table 2). With the small microspheres, the level of uptake ranged from 2 X 110^{-6} moles/ug body wt/h for glycine fo 1.8 X 10^{-3} moles/jig body wt/h for acetate. No particular significance should be attached to these uptake levels due to the high (20 mM) concentrations of substrate used. The significant aspects of this experiment were: (1) that uptake of label occurred only when the label was in particulate form and (2) that there was a greater uptake of the smaller (0.5-10 ¡A) spheres than of the larger (15 u) spheres, suggesting that particle size may be an important factor in food uptake by *R. marina.*

In a classic series of papers, Stephens and co-workers have shown that representatives of most phyla can absorb and assimilate various organic molecules present in sea water in dilute concentrations (Stephens, 1962 a,b, 1964, 1967; Stephens and Schinske, 1961). More recently, Chia and Warwick (1969) observed significant uptake of ^{3}H-labelled glucose from 10^{-5} M solutions by the marine nematodes

TABLE 2

Uptake of latex microspheres by *Rhabditis marina.* Figures indicate number of microspheres ingested per nematode per 24 hours.

Substrate	Microsphere diameter (μ)	
	15 ± 5	0.5-10
Glucose	none	131
Sucrose	none	111
Glycine	10	228
Aspartic acid	13	630
Acetic acid	88	120
Sodium bicarbonate	225	2815

Pontonema vulgare and *Dorylaimopsis punctatus. D. punctatus* was also examined for glycine uptake and, apparently, only took up a small amount of label.

At this point, it is far too early to speculate on the uptake of dissolved organic substances by free-living marine nematodes. Chia and Warwick did not work with axenic nematodes; they did, however, sterilize the sea water used and they were of the opinion that the exposure period (24 h) did not provide enough time for a significant microbial population to develop in their experimental vessels. In our experiments, *Rhabditis marina* neither absorbed dissolved organic matter through its body wall nor did it ingest such material through the gut. If dissolved organic substances do play a role in the nutrition of this organism, they must enter the gut along with the particulate material ingested. From the current observations, it appears unlikely that dissolved organic substances represent a major nutritional pathway for *R. marina.* More likely, dissolved organic substances are absorbed by *R. marina* via a bacterial intermediary; *R. marina,* like other free-living rhabditids which have been studied, feeds extensively on bacteria (Doughertv and Calhoun, 1948; Nicholas, 1962; Tietjen et al., 1970).

Obviously, further experimental work is called for to elucidate the role of dissolved organic substances in the nutrition of marine

nematodes. While axenic cultures may not be absolutely necessary to examine this problem, they, nevertheless, are the only way by which one can be *absolutely* certain that uptake of such substances is by the nematode itself and not via another living intermediary.

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Summary

Monoxenic *Rhabditis marina* were freed from the associated bacteria *(Pseudomonas* sp.) by aseptic washing and transfer through anti-biotic-containing media. *R. marina* was maintained axenically on Grace's Insect media and a marine salt base. The addition of casamino acids, hemoglobin and crude natural materials rich in lipids were found to stimulate growth and reproduction. Lipid rich materials such as TEM (triethyleneamine) and TWEEN 80 were consistently stimulatory, and replaced the amino acid mixture when supplemented with whole egg powder. Mixtures of various fatty acids were able to replace the undefined lipid mixtures. It is suspected that *R. marina* has fewer fatty acid synthetic abilities than other free-living nematodes which have been studied.

The uptake of several dissolved organic substances by *R. marina* was examined. No uptake of ^{14}C-labelled organic substances from solution was observed, even at high (20 mM) concentrations. The addition of small **(0.5-10 μ)** latex microspheres to the experimental medium, however, resulted in uptake of label. This suggests that the major nutritional pathway for *R. marina* is probably through particulate material, such as bacteria and perhaps algae.

REFERENCES

CHIA, F.s. and WARWICK, R.M., 1969. — Assimilation of labelled glucose from seawater by marine nematodes. *Nature,* 224, pp. 720-721.

DOUGHERTY, B.c., 1950. — Some sources are characteristics of the heat-labile nutritional requirement(s) of the nematode, *Rhabditis briggsae. Anat. Record,* 108, pp. 415.

DOUGHERTY, B.c., 1951. — The axenic cultivation of *Rhabditis briggsae* Dougherty and Nigon, 1949 (Nematoda: Rhabditidae). II. Some sources and characteristics of "Factor Rb.", *Exp. Parasitol.,* 1, *pp.* 34-45.

DOUGHERTY, E.c. and CALHOUN, H.G., 1948. — Experiences in culturing *Rhabditis pellio* (Schneider, 1866) *bütschli* 1873 (Nematoda: Rhabditidae), and related soil nematodes. *Proc. helm. Soc. Wash.,* 15, *pp.* 55-68.

DOUGHERTY, E.C, HANSEN, E.L., NICHOLAS, **W.L., MOLLETT, J.A.** and YARWOOD, **E.A.,** 1959. — Axenic cultivation of *Caenorhabditis briggsae* (Nematoda: Rhabditidae) with supplemented and unsupplemented chemically defined media. *Ann. N.Y. Acad. Se.,* 77, *pp.* 176-217.

GERLACH, S.A. and SCHRAGE, M., 1971. — Life cycles in marine meiobenthos. Experiments at various temperatures with *Monhystera disjuncta* and *Theristus pertenuis* (Nematoda). *Mar. Biol.,* 9, *pp.* 274-280.

GBRLACH, S.A. and SCHRAGE, M., 1972. — Life cycles at low temperatures in some free-living marine nematodes. *Veröff. Inst. Meereforsch.* Bremerh., 14, *pp.* 5-11.

GRACE, T.c.c, 1962. — Establishment of four strains of cells from insect tissues grown in vitro. *Nature,* 195, *pp.* 788-789.

HOLTz, G.C., 1964. — Nutrition and metabolism of ciliates. *In* Biochemistry and physiology of Protozoa, S.H. Hutner, Ed. Academic Press, New York, *pp.* 199-242.

HIEB, W.F. and ROTHSTEIN, M., 1968. — Sterol requirement for reproduction of a free-living nematode. *Science*, 160, *pp.* 778-780.

HIEB, w.F., STOKSTAD, E.L.R. and ROTHSTEIN, M., 1970. — Heme requirement for reproduction of a free-living nematode. *Science,* 168, *pp.* 143-144.

HOPPER, B.E., FELL, j.w. and CEFALU, R.c, 1973. — Effect of temperature on life cycles of nematodes associated with the mangrove *(Rhizophora mangle)* detrital system. *Mar. Biol.,* 23, *pp.* 293-296.

HUTNER, **S.H., BAKER, H., AARONSON, S., NATHAN, H.A., RODRIGUEZ, E., LOCKWOOD, S.,** SANDERS, M. and PETERSEN, R.A., 1957. — Growing *Ochromonas malhamensis* above 35 C. *J. Protozool.*, 4, *pp.* 259-269.

LEE, J.J., TETJEN, J.H., **STONE, R.J.,** MULLER, **W.A., RULLMAN, J. and** MC **ENERY, M., 1970.** — The cultivation and physiological ecology of members of salt marsh epiphytic communities. *Helgoländer wiss. Meeresunters.,* 20, pp. l.')6-156.

LOWER, w.R. and BUECHER, J., 1970. — Axenic cuJturing of **nematodes:** an easily prepared medium containing yeast extract. *Nematoloyica,* 16, *pp.* 563-566.

NICHOLAS, w.L., 1962. — A study of a species of *Acrobeloides* (Cephalobidae) in laboratory culture. *Nematoloyica,* 8, *pp.* 99-109.

ROTHSTEIN, M., 1970. — Nematode biochemistry. XI. Biosynthesis of fatty acids by *Caenorhabditis briggsae* and *Panayrellus* ***redivivus. Int. J. Biochem.,*** 1, *pp.* 422-428.

ROTHSTEIN, M., 1974. — Practical methods for the axenic culture of the free-living nematodes *Turbatrix aceti* and *Caenorhabditis briggsae. Comp. Biochem. Physiol.,* 49 B, *pp.* 669-678.

ROLSTEIN, M. and GÖTZ., p., 1968. — Biosynthesis of fatty acids in the free-living nematode *Turbatrix aceti. Arch. Biochem. Biophys.,* 126, *pp.* 131-140.

ROTHSTEIN, M. and NICHOLAS, w.L., 1969. — Culture methods and nutrition of nematodes and Acanthocephala. In Chemical Zoology III, Echinodermata, Nematoda and Acanthocephala, M, Florkin and B.T. Scheer, Eds. Acad. Press, N.Y., *pp.* 289-328.

SAYRE, F.w., HANSEN, E.L. and YARWOOD, E.A., 1963. — Biochemical aspects of the nutrition of *Caenorhabditis briggsae. Exp. Parasitol.,* 13, *pp.* 98-107.

SHORB, M.S. and LUND, P.G., 1958. — Requirement of *Trichomonas* for unidentified growth factors, saturated and unsaturated fatty acids. J. *Protozool,* 6, *pp.* 122-130.

STEPHENS, G.c, 1962 a. — Uptake of organic material by aquatic invertebrates. I. Uptake of glucose by the solitary coral, *Fungia scutaria. Biol. Bull.,* 123, *pp.* 648-659.

STEPHENS, G.c, 1962 b. — Uptake of amino acids by the bamboo worm, *Clymenella torquata. Biol. Bull.,* 123, p. 512.

STEPHENS, G.C., 1964. — Uptake of organic material by aquatic invertebrates. **II.** Uptake of glycine by brackish water annelids. *Biol. Bull.,* 126, *pp.* 150-162.

STEPHENS, G.C., 1967. — Dissolved organic material as a nutritional source for marine and estuarine invertebrates. *In Estuaries,* G.H. Lauff, Ed. AAAS Publ. 83, Washington, *pp.* 367-373.

STEPHENS, G.C. and SCHINSKE, R.A., 1961. — Uptake of amino acids by marine invertebrates. *Limnol. Oceanogr.,* 6, *pp.* 175-181.

TIETJEN, J.H. and LEE, J.J., 1972. — Life cycles of marine nematodes. Influence of temperature and salinity on the development of *Monhystera denticulata. Oecologia,* 10, pp. 167-176.

TIETJEN, J.H. and LEE, j.j., 1973. — Life history and feeding habits of the marine nematode, *Chromadora macrolaimoides* Steiner. *Oecologia,* 12, *pp.* 303-314.

TIETJEN, J.H. and LEE, J.J., 1975. — Feeding behavior of marine nematodes. *In* Ecology of marine benthos, B.C. Coull, Ed. Univ. of South Carolina Press, Columbia, S.C., In press.

TIETJEN, J.H., LEE, J.J., RULLMAN, J., GREENGART, A. and **TROMPETER, J.,** 1970. — Gnotobiotic culture and physiological ecology of the marine nematode *Rhabditis marina* Bastian. *Limnol. Oceanogr.,* 15, *pp.* 535-543.

TOMLINSON, G. and ROTHSTEIN, H., 1962. — Nematode biochemistry. I. Culture methods. *Biochem. Biophys. Acta,* 63, *pp.* 465-470.

VAN FLETEREN, J.R., 1973. — Amino acid requirements of the free-living nematode, *Caenorhabditis briggsae. Nematologica,* 19, *pp.* 93-99.

VAN FLETEREN, J.R., 1974. — Nematode growth factor. *Nature,* 248, *pp.* 255-257.