MARINE ECOLOGY

A Comprehensive, Integrated Treatise on Life in Oceans and Coastal Waters

Editor
OTTO KINNE

VOLUME III
Cultivation
Part 3

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FOREWORD

to

VOLUME III: CULTIVATION

'Cultivation' reviews the information which has accumulated on our present capacity for supporting marine micro-organisms, plants and animals under environmental and nutritive conditions which are, to a considerable degree, controlled. The volume is subdivided into three parts, containing the following chapters*:

Part 1

Chapter 1: Introduction to Volume III
Chapter 2: Cultivation of Marine Organisms: Water-quality Management and Technology
Chapter 3: Cultivation of Micro-organisms
Chapter 4: Cultivation of Plants

Part 2

Chapter 5.1: Cultivation of Animals—Research Cultivation

Part 3

Chapter 5.11: Axenic Cultivation
Chapter 5.2: Commercial Cultivation (Aquaculture)
Chapter 6: Multispecies Culture and Microcosms
Chapter 7: Chemical Contamination of Culture Media: Assessment, Avoidance and Control

We have made every effort to present comprehensive reviews, covering essential aspects of the cultivation of marine organisms. It soon became apparent, however, that only in a few cases, comparative, critical assessments of different culture methods and technologies were possible. Many publications suffer from insufficient detail, or even total lack of information regarding source, environmental history and nutrition of the organisms cultivated or the culture method employed. Exact data on environmental factors—such as light, temperature, salinity or dissolved gases—and on diet are absolute requirements for proper evaluation of the results presented. No less important are the origin of the organisms concerned, culture-water quality and technological aspects.

Culture methods are often an outcome of empiricism and intuition. A technique is tried, and if it works, the investigator sticks with it, rationalizing only afterwards the reasons for its application and success. The factors truly critical to success have

* See Editorial Note, p. vi.

rarely been pinpointed. Some portions of the reviews presented must, therefore, remain tentative, descriptive or pragmatical.

Cultivation is not an end in itself. It serves as a means to solve specific research problems. Due to the large variety of problems and the overwhelming diversity of marine life, a multitude of different culture methods have been developed. In fact, concepts, goals and techniques applied in cultivation diverge more than in other branches of marine ecology.

Most experiments conducted on marine organisms involve elements of cultivation. Micro-organisms, crustaceans, molluscs and fishes, for example, have been maintained, reared or bred in thousands of experiments. It was neither possible nor desirable to consider all publications in detail. We have attempted to settle the conflict between our intention to present comprehensive accounts and the need to avoid undue repetition by tabulating the information at hand or by referring to pertinent books or reviews.

I acknowledge with pleasure the support, advice and criticism received from the contributors, as well as from Drs. D. F. Alderdice, J. R. Brett, H. P. Bulnheim, G. Persoone, A. Gaertner and D. Siebers. Additional supporters are mentioned at the end of the respective chapters. The assistance of M. Blake, V. Clark, J. Marschall, H. L. Nichols, I. Schritt and H. Witt is deeply appreciated.

O.K.

Editorial Note

The two chapters originally envisaged to comprise Part 3 of Volume III—Diseases of Plants and Diseases of Animals—will not be published in this form. Together with a general introduction, Chapter 9 will appear in a separate two-part book:

O. KINNE (Ed.) *Diseases of Marine Animals*, Wiley, London.

The reasons for this change in our original concept are (i) the fundamental importance of animal diseases not only for cultivation, but also for proper ecological assessment of both distribution and performance of marine organisms; (ii) the large amount of information available on diseases of marine animals; (iii) the rather restricted information presently at hand on diseases of marine plants.

O.K.

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OF
VOLUME III, PART 3

5. CULTIVATION OF ANIMALS

5.1 RESEARCH CULTIVATION

5.11 AXENIC CULTIVATION

L. PROVASOLI

(1) Introduction

Axenic cultivation originated with microbiology. The progress of this art along with the use of microbes in quantitative biology, and application of this type of cultivation to other organisms (or their parts, tissues), were due to the possibility of controlling the homogeneity and purity of biological material. Technical complexities have limited the use of axenic cultures of higher animals. Complexity of organisms, diversity of foods and of environmental conditions needed, all intensify the difficulty of providing axenic conditions suitable for growth and reproduction. The usual difficulties in devising complex artificial diets for insects, domesticated animals, and fishes are exacerbated by the necessity of asepsis and even more so when one needs to control chemical purity (i.e. chemically defined diets) as well as organismal purity.

Yet in approaching or achieving these two wholly unnatural conditions, information emerges, paradoxically, of ecological importance.

The use of chemically defined media and axenic cultures of phytoplankters, by revealing the essentiality of some vitamins, trace metals, and other nutrients, has added ecological determinants and permitted studies on rates of uptake and utilization of nutrients, and on synthesis and excretion of metabolites (p. 616). The ecological relevance of nutritional data on these producers is obvious because these organisms are osmotrophic and photosynthetic; hence their activity depends on the chemical and physical environmental variables.

The inherent phagotrophic peculiarities of animals complicate the analysis of nutritional components; growth now depends largely upon the availability, abundance, and chemical composition of the prey—a relationship that becomes more tangled as the food web becomes longer and more varied. Predation, mutualism, symbiosis, and parasitism need to be defined at the nutritional level and one can even consider the possibility that the seasonal variations in prey species, with their differences in chemical compositions, may affect growth rate, sexuality, and fertility of the predator.

The nutritional meaning of these relationships, and interdependencies of organisms can be discerned by combining axenic partners and by reconstructing defined, and increasingly complex biocenoses. Unexpected and subtler relationships between partners may then emerge, e.g., probiotic or antibiotic effects, either direct or mediated by exocrines, pheromones (p. 616), and other excreta in the medium.

The chemical definition of the essential nutritional requirements of the predator may be used to relate the nutritiousness of prey with its content of essential nutrients for the predator, and has been the basis for the exceedingly efficient diets for commercially important species (notably poultry, trout, silkworm, and other insects).

Few marine animals are in axenic culture and the results obtained are often preliminary. Because of the incompleteness of information, reference will often be made to freshwater species, or to phylogenetically related groups, in an effort to set the available information in a wider context.

(2) General Procedures

(a) Axenization

Initial axenization requires elimination of foreign organisms. This can be achieved by physical and chemical means, or their combination. Before the advent of antibiotics, axenization relied on diluting out the unwanted accompanying organisms while keeping constant or augmenting the number of organisms to be purified.

Mild, repeated centrifugation and use of nets, filter papers, and membranes of various porosity can eliminate most contaminants whose size or specific gravity substantially differ from the organism to be axenized. Following this preliminary sorting, organisms ranging from 10 to 1000 μm (protozoans, young larval forms) are collected under the dissecting microscope with a Pasteur pipette and transferred into sterile sea water. To avoid injury and to reduce to a minimum the liquid transferred with the organism from one bath to another, the bore of the pipette should be 4 to 6 times larger than the dimension of the organism to be transferred. Details on making micropipettes have been presented in DROOP (1969). The volume of sterile fluid in each bath should be proportional to the size of the organism to facilitate locating the organisms: 1 drop on a slide for 5 to 10 μm organisms (LWOFF, 1929); 0·5 to 1 ml in depression slides for 20 to 100 μm organisms; 2 to 10 ml in watch glasses, crystallizing dishes, or small Petri dishes for larger organisms. The number of sterile baths needed depends on the dilution factor (i.e. the volume of liquid carried with organisms and the volume of the bath [1 : 10–1 : 100] and the number of organisms to be diluted out [i.e. 10^6–10^{12} bacteria.ml^{-1} in heavily infected cultures]).

Pipette washing is rewarding but tedious; a continuous dilution apparatus may be appealing (CLAFF, 1940). Behavioural habits or tactism may be profitably used to find easier and speedier dilution methods; e.g. 1-m-long migration tubes, V-shaped tubes for anaerobes (GLASER and CORIA, 1930), electromigration (SOLDO and VAN WAGTENDONK, 1971; p. 218).

Elimination by dilution of prey organisms larger than a few microns is easy; elimination of micro-organisms (bacteria, yeast, mould spores) is more difficult because undigested micro-organisms and the normal gut microflora of invertebrates have also to be eliminated. A high proportion of axenic protozoans can be obtained by alternating 4 series of 5 to 7 rapid baths with 3 prolonged baths. The rapid baths (5–10 mins each) serve to dilute out the contaminants; a prolonged bath (30–60 mins) allows emptying of vacuoles (ciliates) and gut. Serial washings have been used

to axenize freshwater crustaceans (STUART and co-authors, 1931) and species of *Tigriopus* (PROVASOLI and co-authors, 1959).

By combining serial washings with antibiotics the number of washings can be reduced and success increased. The effectiveness of antibiotics has often been overrated because in some cases axenization was obtained by using simply penicillin + streptomycin. Judging from many personal communications, failures far exceeded success; failures are seldom reported in print. A familiar complaint is that the same antibiotic treatments may succeed or fail under ostensibly identical conditions. This is not surprising because the bacterial flora varies widely in composition and size with seasons, localities, and biocenoses (SIEBURTH, 1967).

The uncertainty of results reflects also the properties of antibiotics: most antibiotics are bacteriostatic, inhibit a limited bacterial spectrum, were screened and hence were most effective against human pathogens. Since the marine microflora is poor in Gram-positive and rich in Gram-negative bacteria and since many Gram-negatives resist most antibiotics, mixtures of wide-spectrum antibiotics offer better prospects. Penicillin seems to inhibit quite adequately most marine Gram-positives and is generally non-toxic in high concentration (1–2·000 U ml^{-1}) to protozoans and invertebrates. Combinations of streptomycin, tetracyclins, chloramphenicol, polymixin B, and neomycin are used to inhibit the Gram-negatives; these four antibiotics are often more toxic to invertebrates than are penicillin + streptomycin and are used at 20 to 100 $\mu g\ ml^{-1}$. Sulpha drugs are also quite useful against Gram-negatives; KANAZAWA (1968) reported that a mixture of sulphamerazine, sulphisomidine, sulphisoxazole, and homosulphanilamide was very helpful in axenizing several seaweeds (10-min treatment at 0·02% for each sulpha drug with shaking, followed by cultivation for 15–30 days in the presence of 0·005% of each; the medium was changed every few days; see also Chapter 4.1).

Since antibacterial antibiotics are bactericidal only in high concentrations and are most active against dividing bacteria, it is advisable to use the highest concentration tolerated by the animals for a short period (overnight) in a medium favouring limited bacterial growth thanks to the addition of small quantities of peptones, yeast, or liver extracts (0·1–1 mg%) and sugars (1–5 mg%). The surviving animals are washed several times the morning after and inoculated in separate tubes of media favouring growth. Alternatively, lower doses of antibiotics are added also to the growth media.

Incomplete axenization often results in dense growth of the surviving contaminants, which are no longer restrained by competitors. Most obnoxious are yeast and fungi because the antifungal antibiotics are poorly soluble and often quite toxic. Fungizone, mycostatin (nystatin) (LEE and co-authors, 1970) and candicidin (PROVASOLI and GOLD, 1962) were successful but only after prolonged treatments. It is therefore advisable to avoid fungal or yeast contamination by washing the organisms repeatedly as soon as possible after collection from nature. Residual bacterial infections can be eliminated by testing their sensitivity to antibiotics, by plating the supernatant on nutrient agar, and using the Baltimore Biological Laboratory Sensidiscs, and so selecting the most active antibiotic or sulpha drug.

Some stages of animals are easier to axenize than others. Resting stages, such as cysts of protozoans, durable eggs of *Artemia salina*, and many eggs, are clad in poorly permeable coats, hence strongly bactericidal solutions can be used to

sterilize their surface. Short immersions in solutions of $HgCl_2$ or Merthiolate, followed immediately by ample dilution of the poison by a continuous flow of sterile sea water in closed receptacles with a filter pad at the bottom or repeated centrifugations have been successful. Motile and non-feeding stages, such as freshly hatched larvae and planulae of coelenterates, are easy to purify by washings, as are animals right after a moult because their surfaces are almost aseptic (PROVASOLI and co-authors, 1959).

(b) Sustenance Media

Only in very rare instances can one rear the axenized organism directly on artificial food, let alone chemically defined media. Obviously, direct transfer to artificial media should be attempted only when enough information is available on the food requirements of closely related species or of animals phylogenetically unrelated but feeding or preying on the same type of food. This information is rarely available for marine animals since few have been grown axenically.

A convenient approach is to aim at an intermediate step: that of growing the purified animals on living food (i.e. gnotobiotic cultures, the food organisms being mono- or dixenic, if possible). For animals feeding on micro-organisms and small algae, gnotobiotic cultures can be obtained either by eliminating the contaminants gradually or by feeding the axenized predators with axenic cultures of food organisms.

Several gnotobiotic cultures of foraminiferans and ciliates were thus obtained, even monoxenic cultures of *Allogromia* sp. (LEE and PIERCE, 1963) and of *Uronema marinum* (LEE and co-authors, 1971b) fed on one bacterial species. The availability of many axenic cultures of marine unicellular algae permitted several cultures of herbivorous crustaceans (p. 742), and should allow similar cultures of the herbivores belonging to other marine phyla. The sea squirts are good candidates for mono- or dixenic cultures since they grow well in agnotobiotic cultures and have been used for genetic investigations by SABBADIN (1971).

The advantage of this intermediate step is the availability of permanent cultures from which it is possible to obtain, with less effort, a continuous supply of axenic animals for inoculating a variety of artificial media. This goal is facilitated by selecting food organisms which are easy to eliminate: either by using an antibiotic to which they are particularly sensitive (bacteria) or by transferring the cultures to non-nutrient media kept in the dark, followed by a few washings (algae); if obligate phototrophic algae are chosen, darkness alone will suffice (DROOP, 1970; MCGINN, 1971). The intermediate step of monoxenic cultures can be avoided only in particularly favourable circumstances: the durable eggs (cysts) of *Artemia salina* are commercially available and resist bacteriocidal agents; an aseptic inoculum is thus readily obtained.

Devising an artificial medium is often an art, since representatives of many phyla or orders, even non-marine, have never been cultured. Guidance in finding crude materials rich in diverse nutrients can be derived from the literature on nutrition. Particularly useful are the symposia edited by DOUGHERTY (1959) and KINNE and BULNHEIM (1970) on cultivation of invertebrates, and the book by TAYLOR and BAKER (1968) on cultivating parasites. Inspection of the commercial

diets employed for pets, poultry, rats, trout, and the compilation of insect media (HOUSE and co-authors, 1971) will suggest a variety of nutrient sources.

The goal at this stage is to provide a 'complete' medium despite ignorance of specific requirements. In broad lines the essential nutrients are: minerals and trace elements, sources of energy and carbon (carbohydrates, fats), special building blocks (amino acids and nucleic acids), water and fat-soluble vitamins, lipids, sterols, and rarely, hormones. The raw materials often used include refined and unrefined proteins (skim milk, casein, albumen, fish protein, etc.), sugar, starch, crude RNA and DNA, blood serum, corn oil, crude lecithins, animal or vegetable sterols, blood, various yeast and liver preparations (extracts, homogenates, autolysates) and other vitamin-rich preparations.

Equally important—but often neglected—are the ratios of nutrients (proteins/carbohydrates/fats), appetite factors, the physical attributes of the diet (size, consistency) and possible toxicity of organic solutes.

(3) Gnotobiotic Cultures of Invertebrates

As mentioned, the first step toward achieving an axenic culture is to obtain continuously reproducing cultures of a predator on a restricted number of identified prey organisms. Gnotobiotic cultures and axenic species can be combined to form a planned artificial biocenosis to study the interactions between various species and to acquire information on population dynamics, predation, etc. (Chapter 6). Fortuitous, but often equally useful, are gnotobiotic cultures derived from the progressive elimination of accompanying species in the course of obtaining axenic cultures. Information on the kind of organisms that represent the actual or potential food for benthic foraminiferans was thus acquired by LEE and co-authors (1966) by tagging species of algae with ^{14}C and measuring the radio-activity of the predator—a rapid and sensitive method for sorting out the key prey species. The components of an extremely varied microflora, which were the important food organisms for foraminiferans, were thereby identified. Such an analysis leads also to simplification of the biocenosis by retaining only those prey species which contribute most to the growth of the desired species. When the bacterial components were eliminated it became apparent that the algal food, alone, failed to support continuous reproduction of 4 species of foraminiferans: 1 or 2 species of bacteria were necessary to provide essential nutrients not present in the algal food (MULLER and LEE, 1969).

The role of bacteria and other micro-organisms as food for protozoans was known long ago but hints are accumulating that bacteria perform other functions: e.g., some species of bacteria induce or repress sexuality in ciliates (CHATTON and CHATTON, 1925). The role of bacteria in invertebrate nutrition is largely unexplored.* In a thoughtful review on the nutrition of zooplankton EDMONDSON (1957) asks whether bacteria are utilized as food—an issue too often ignored by other ecologists. Similar gaps, in the consideration of all the significant variables involved, seem to beset mathematical modelling of the environment—brilliant mathematics can hardly compensate for poor ecology.

* Metamorphosis in the cnidarian *Hydractinia echinata* has been shown to be induced by bacteria (MÜLLER, 1969; see also p. 659).

In the process of axenizing, one often stumbles on unexpected effects caused by the elimination of one or more of the members of a natural community. In defining the nutritional value of different algae to *Tigriopus japonicus*, *Platymonas tetrathele* ('*Platynonas* No. 5') proved to be an incomplete food: it allowed only six generations of *T. japonicus*. Since *T. japonicus* had been maintained for years on *P. tetrathele* as the sole alga in bacterized cultures, the unidentified bacteria supplied the missing nutrients (no yeasts or other non-bacterial micro-organisms were present).

The nutritive value of algae for predators varies widely among algal species: some algae do not support growth of *Tigriopus* species to adulthood; some support growth to midget, infertile adults; and some to normal size, fertile adults (PROVASOLI and co-authors, 1959). Only rarely is one algal species a 'complete' food, i.e. permitting an indefinite number of generations. Similar results were obtained for a marine amoeba, *Oxyrrhis marina* and a rotifer by DROOP (1966) who gives a table on the nutritional value of 30 algae for monoxenic and agnotobiotic cultures of 12 predators. More often, two species of algae or more are needed to supply all the nutritional requirements of the predator, indicating that varied food is a safer way to satisfy all nutritional needs; algal blooms, constituted almost solely of one species, may often be an incomplete food.

However, the nutritional deficiencies of single species of algae for growing *Tigriopus japonicus* can also be relieved by adding vitamins to the culture medium (SHIRAISHI and PROVASOLI, 1959). This is apparently not an isolated phenomenon; vitamins and crude organic extracts relieved the nutritional deficiency caused by subnormal salinity stress of *Artemia salina*, fed on two algae (D'AGOSTINO and PROVASOLI, 1968). Twelve species of freshwater cladocerans could be grown indefinitely on one species of alga, *Chlamydomonas reinhardtii*, by MURPHY (1970) by adding a B vitamin mixture to the medium in which the crustacean and the alga were grown together. A definite nutritional hierarchy was found by MURPHY; some daphnids could be grown only when either the concentration or number of vitamins were higher.

Whether the vitamins and/or organic additions were beneficial directly to the crustacean or *via* algal uptake, was settled by growing *Chlamydomonas reinhardii* and *Scenedesmus obliquus*, the food organisms used for *Daphnia magna*, separately in a medium with and without vitamins and then supplying them to *D. magna* grown in a medium without vitamins. Only the algae grown in vitamins (not needed by *C. reinhardtii* and *S. obliquus*) permitted an indefinite number of generations of *D. magna*.

Hence, the nutritional value of the algae may be affected by the organic solutes present in waters (D'AGOSTINO and PROVASOLI, 1970). Conversely, organic enrichments may be very useful in obtaining continuous fertility of predators fed on algae or other prey in aseptic conditions.

These preliminary results indicate that at least some basic techniques are available to bring into gnotobiotic culture the marine organisms which can be reared in the laboratory on living prey (see also Chapter 5.1).

Newborn larvae of *Ostrea edulis* were disinfected with antibiotics and were grown monoxenically to settling stage by feeding them on *Monochrysis lutheri* (MILLAR and SCOTT, 1967). Indefinite monoxenic culture of the marine acoel turbellarian

Parotocelis luteola was obtained by KOZLOFF (1969) with either *Nitzschia dissipata* or *Navicula pavillardi* as food organisms; these diatoms were the only ones out of 12 species of diatoms tried which would support growth of the worm. Another acoel, the symbiotic *Convoluta roscoffensis*, can be grown monoxenically (PROVASOLI and co-authors, unpublished) in sterile sea water supplemented with nitrates, phosphates, trace metals, and 3 vitamins (ES enrichment; PROVASOLI, 1968) indicating that bacteria are not necessary for growth. A simple medium sufficed for *C. roscoffensis* because its symbiont, *Platymonas convolutae*, a photosynthetic chlorophyte, synthesizes and supplies *C. roscoffensis* with all nutrients needed. Some artificial symbionts belonging to the genus *Prasinocladus* performed the same feat, but several species of *Platymonas* were unable to do so (PROVASOLI and co-authors, 1968).

(4) Axenic Cultures of Protozoa

The free-living protozoans comprise an array of 'acellular' organisms of diverse origins; they range from non-photosynthetic flagellates, to Amoebae, Acantharia, Foraminifera, and ciliates. Nutritionally, most of them feed on micro-organisms and unicellular algae, but some feed on other protozoans or small larval forms of invertebrates. They share the same prey organisms with many filter-feeders and could be similarly lumped into the 'herbivores'—an ambiguous term borrowed from land food-chains which poorly defines the place of these organisms in the aquatic food web. Protozoans differ clearly from the filter-feeders in their varied ways of acquiring the same type of prey (Volume II: PANDIAN, 1975); thus, they can capture their food in locales unsuited to most filter-feeders.

Feeding on the same type of prey is not necessarily equivalent or synonymous with having the same chemical nutritional requirements. Exogenous nutritional requirements represent synthetic disabilities which seem to be mainly connected with phylogenetic position. Arranging the chapter subheadings in taxonomic order facilitates the distinction between general and group-specific nutritional requirements and comparisons between phylogenetic groups. As we will see, while phagotrophy is apparently the 'natural' way of feeding, many protozoans may display remarkable osmotrophic abilities—a physiological vestige presumably inherited from their osmotrophic, photosynthetic ancestors (see also Chapter 5.1).

(a) Colourless Flagellates

Three marine dinoflagellates have been grown axenically in defined media and two choanoflagellates on semi-defined media. Their nutritional requirements illustrate, albeit sketchily, the transition from photo-autotrophy to osmotrophic or phagotrophic heterotrophy.

Crypthecodinium cohnii (syn.: *Gyrodinium cohnii*)

Crypthecodinium cohnii is a small dinoflagellate which often abounds where species of *Fucus* decay (PRINGSHEIM, 1956). BIECHELER (1952) reports that the

swimming stage can trap *Bodo* sp., bring it near the flagellar pore and suck the cytoplasm of the prey within a few seconds. JAVORNICKY (1962) considers the swimming stage of *C. cohnii* a zoospore since no division occurs in this stage. Two or more divisions take place in the non-motile vegetative phase which lacks openings in its cell wall. Therefore, osmotrophy seems to prevail in acquiring nutrients.

Several strains of *Crypthecodinium cohnii* have been isolated and grown axenically in artificial media. The Massachusetts' strain (G.C.) (PROVASOLI and GOLD, 1962) and the Puerto Rico strain (P.R.) (GOLD and BAREN, 1966) were axenized by washings or with the help of antibiotic and antifungal mixtures and grown first in yeast digest (PRINGSHEIM, 1956) and acetate. They grow luxuriantly in simple defined media. Both are euryhaline (good but slow growth occurring even at 0·3 and 4% NaCl) and have a pH latitude unusual for a marine organism (optimum pH 5·7–7·2). They use ammonium salts as N sources; the G.C. strain—after isolation in a medium containing peptone and yeast digest—required histidine as N source (other amino acids were not utilized) but could be trained to grow in ammonium salts. The P.R. strain seems to utilize also glutamic acid and alanine at optimal temperatures (20°–28° C). Much better growth of the G.C. strain was obtained with a mixture of ammonium sulphate and histidine + betaine (other amines are also utilized). High phosphate, glycerophosphate, and nucleic acids are all a good source of phosphorous. *C. cohnii* utilizes a variety of carbon sources: glucose and glycerol are the best single C sources; ethanol, acetic and other fatty acids, succinic and fumaric were less effective when added alone; but a combination of glucose or glycerol with organic acids gave optimal growth. Biotin is an absolute requirement; thiamine is highly beneficial, but continuous low-density growth (20 transfers) was obtained in the absence of thiamine.

The only lack of versatility of *Crypthecodinium cohnii* is in response to temperature. Optimal growth for the G.C. strain occurred at 20° to 28° C; growth was inhibited at 15° to 20° and 30° C; no growth was obtained at 10° C or above 30° C. The P.R. strain had an optimum at 30° C and was inhibited at 20° and 35° C. To relieve the inhibition at 35° C, addition of tryptophan and proline and vitamin B_{12} was indispensable (GOLD and BAREN, 1966). New nutritional requirements at sublethal temperatures were similarly found in *Euglena gracilis* and *Ochromonas malhamensis* (HUTNER and co-authors, 1957) and may be of ecological importance (PROVASOLI, 1958), i.e. polluted waters may extend the geographical distribution to warmer waters.

The ability of *Crypthecodinium cohnii* to utilize several N, P, and C sources in defined media and to grow better in mixtures of nutrients, as well as its tolerance to pH, accords well for an organism thriving in an environment rich in a variety of degradation products. The PRINGSHEIM and MCLAUGHLIN strains cannot be grown in this defined medium and grow but poorly in a peptone-yeast digest-glucose-acetate medium, indicating additional undefined requirements. The sensitivity toward biotin can be used to assay this vitamin in sea water (PROVASOLI and GOLD, 1959). The ability to grow on agar (KELLER and co-authors, 1968) and the sexuality found in our strain by C. K. FRANKER (personal communication), coupled with a good growth potential (4 millions ml^{-1} in 7 days), make of *C. cohnii* a very promising organism for biochemical research.