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Title: Heterotrophic Bacteria Associated with a Feed Algae for Oyster Larvae

Abstract approved: Dr. Jong S. Lee

Aerobic heterotrophic bacteria associated with oyster larvae feed algae Isochrysis galbana, Monochrysis lutheri and Pseudoisochrysis paradoxa were isolated and enumerated. The bacterial numbers ranged from 7.8×10^3 to 3.9×10^6 CFU per ml. The bacteria associated with Pseudoisochrysis sp. were identified and the majority of isolates belonged to genera Leucothrix (51%). Also present were members of Pseudomonas III sp. (19.1%), atypical Moraxella sp. (16.8%), Moraxella sp. (7.2%) and Flavobacterium sp. (5.9%).

The growth of bacteria on marine agar was fastidious and took four days to form visible colonies. None of the bacterial isolates grew in buffered salt broth in which the algae had been grown. Marine broth supplemented with 0.1% beef extract best supported the growth of the isolates, while ferric citrate (3×10^{-4} M) supported their growth in buffered salt broth.

The role of algae as a solid support for bacteria was investigated by studying the attachment of bacteria on glass slides suspended in the growth medium. The percent of Leucothrix sp. attached to the slide was 2.1-3.0%. This was four to six times greater than that of Staphylo-

coccus (0.5%), an organism well known for its commensal growth on skin and mucus membranes of man and animal.

Hydrophobic attraction, which is thought to play an important role in the orientation of bacteria to solid surfaces, was studied by measuring the adsorption of bacteria on hydrocarbons introduced to a bacterial suspension. Leucothrix sp. exhibited the strongest affinity, whereas Flavobacterium sp. and Pseudomonas sp. adsorbed the least.

An attempt to obtain an axenic culture of algae by antibiotic treatment was unsuccessful due to the detrimental effect of antibiotics to both algae and bacteria.

Heterotrophic Bacteria Associated with a Feed Algae for Oyster Larvae

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INTRODUCTION

Hatchery propagation of oyster larvae commonly depends on unicellular algae as a food source. For some unexplained reason, a batch of algae may occasionally fail to support normal growth and development of oyster larvae. The reason for this loss in food value is not known, but a change in microbial flora of algae was suggested as a probable cause (Breese, W.P., Personal Communications).

The purpose of this investigation was to isolate, identify and examine the bacteria associated with algal cultures. Microorganisms were identified to the genus level and the growth requirements and kinetics of selected isolates were determined. The abilities of bacteria to attach on solid surfaces and hydrophobicity were measured to assess their roles in commensial growth with algae.

LITERATURE REVIEW

Diet for Oyster Larvae

The Pacific oyster, Crassostrea gigas, is the major species raised on the West Coast. The oyster seed is imported from Japan since Northwest bay waters are usually too cold to permit successful spawning of this oyster. But, because of high cost and diminishing supply of Japanese seed, oyster hatcheries have been started to rear larvae. Oyster hatcheries are now producing seed from Crassostrea gigas as well as other species of commercial potential (Breese, 1973; Kolbe et al., 1979). These hatcheries generally use unicellular algae as a food source for the larvae. The unicellular algae commonly used for feed are Pseudoisochrysis paradoxa, Isochrysis galbana, and Monochrysis lutheri (Dupuy, 1973).

The conventional technique used is to grow batches of algae to a suitable density, and transfer the algae to the rearing tanks (Kolbe et al., 1979). Most studies of bivalve feeding have been to determine the algal species and algal densities that would provide optimum growth of the larvae (Loosanoff and Davis, 1963; Walne, 1970; Ukeles, 1971; Langton and McKay, 1976; Epifanio and Ewart, 1977). These studies indicated that diets composed of 3-4 species of algae generally supported faster growth than single species diets.

The relationship between food value of algae and its chemical composition is unclear (Castell and Trider, 1974). Epifanio (1979) fed juvenile Crassostrea virginica one of two diets composed of various

mixtures of four species of algae. Growth of the oyster depended on the presence or absence of particular algal species in the diets and did not correlate with gross chemical composition (protein, lipid, carbohydrate, ash) or amino acid composition of the diets. Other nutritional studies have been performed on adult oysters (Castell and Trider, 1974; Flaak and Epifanio, 1978) and on larval oysters (Helm et al., 1973; Holland and Spencer, 1973; Chu and Dupuy, 1980).

Various explanations for differing food value of algae have been made. Walne (1970) suggested that food value was not related to amino acid composition per se, but possibly to digestibility of algal protein. He also considered the possibility that nutritionally inadequate algae may lack some trace constituents, or that oysters might be unable to destroy the cytoplasmic boundaries of some algae. Others suggested that the difference in food value was directly attributable to differences in the quantity or toxicity of their metabolites (Davis and Guillard, 1957).

Bacteria as Food for Oyster Larvae

The role of bacteria in the nutrition of aquatic animals has been the subject of numerous studies (Gayevskaya, 1938; ZoBell and Feltham, 1938; ZoBell, 1946; Rodina, 1946, 1949; Zhukova, 1963; Jorgensen, 1966; Sorokin, 1968; Fenchel, 1971; Boucher and Chambroux, 1976). It has been shown that bacteria played an important role in the diet of such animals as the harpacticoid copepod Tigriopus californicus (Pravasoli et al., 1959), some marine oligochaetes (Giere, 1975) and the mullet

Mugil cephalis and prawn Metapenaeus bennettae (Moriarty, 1976). Furthermore, it has been shown that some marine animals can subsist solely on a diet of bacteria. These animals include the mussel Mytilus californianus (ZoBell and Feltham, 1938), two species of marine sponges (Reiswig, 1975) and the harpacticoid copepods Tisbe holothuriae and Paramphiascella vararensis (Rieper, 1978). As yet little is known about the role of bacteria in oyster larvae nutrition. Cole (1936) reported that impure cultures of a unicellular algae could support satisfactory growth of oyster larvae, but pure cultures lacked a factor essential for growth. Iami and Hatanaka (1949) used a colorless algae that was cultured on a bacterial diet and fed to the oysters. Since bacteria as well as algae were fed, the role of bacteria on oyster nutrition was not determined.

Bernard (1974) demonstrated that the Pacific oyster (Crassostrea gigas) did not discriminate between algae and bacteria. He found that purple sulfur bacteria were retained and readily digested when presented as pure cultures. When both the bacteria and the algae Chlorella were offered to the oyster, the bacteria rapidly lysed in the stomach while the algae passed almost undigested through the gut and produced bright green pseudo faeces.

The unequivocal proof of importance of bacteria to shellfish nutrition came from the hot water vents on the deep ocean floor. Huge colonies of clams thrived around the vent existing solely on the diet of hydrogen sulfide bacteria (Ballard and Grassle, 1979).

Bacteria as Inhibitor of Oyster Larval Growth

Although numerous studies have claimed that bacteria is an important part of the diet of various marine animals, it is possible that some bacteria could be detrimental to the growth of oyster larvae. A team of scientists at the U.S. Bureau of Commercial Fisheries reported that many mortalities in oyster larval cultures were caused by bacteria, and proposed the use of ultraviolet-treated seawater in all larval cultures (Loosanoff, 1954; Davis and Chanley, 1956). Another Bureau scientist demonstrated that certain bacteria produced toxins that could retard growth of larvae or kill them (Guillard, 1959). A study on the artifical rearing of the oyster larvae Pinctada maxima found that the larvae grew poorly when fed algae contaminated with bacteria. When antibiotics were added to the rearing jars, the bacteria were eliminated and the larval growth resumed (Minaur, 1969).

The Relationship Between Bacteria and Algae

After examination of the role of algal extracellular products on bacterial growth, Bell et al. (1974) suggested that algal blooms created a "phycosphere" under the influence of which microbial activity was altered from that of the surrounding milieu. This hypothesis was based on two observations: (1) marine phytoplankters produce extracellular products, including simple organic compounds (Hellebust, 1965; Fogg, 1966; Thomas, 1971); and, (2) bacteria are basically opportunists, their greatest activity occurring where there is local enrichment of nutrients. Other researchers have suggested that a mutually

beneficial relationship exists between algae and bacteria; the bacteria utilize the algal extracellular products and produce substances such as vitamins which enhance algal growth (Jolley and Jones, 1977; Shiba and Taga, 1980). This relationship is exploited in the use of algae as a means of wastewater treatment. The algae utilize carbon dioxide and the nutrients produced by bacteria through aerobic degradation of organic matters. The algae subsequently release oxygen by photosynthesis. This oxygen is utilized by bacteria to mineralize nitrogen and phosphorus, thereby bringing the nutrient recycling process to near completion (Oron et al., 1979).

Attachment of Bacteria to Solid Surfaces

The attachment of bacteria to solid surfaces is of great importance in many environments, including soil (Marshall, 1976; Burns, 1979), teeth (Gibbons and van Houte, 1975; Rutter, 1979), mammalian epithelial tissues (Jones, 1977) and marine environment (Corpe, 1970; Marshall et al., 1971; Fletcher, 1979). In marine environment, bacteria attached to submerged surfaces could contribute to costly fouling and corrosion of man-made structures (ZoBell and Allen, 1935; Corpe, 1970). In addition to inanimate surfaces, submerged living organisms provide sites for bacterial colonization, such as animal surfaces (Sieburth, 1979) and those of plants.

During the last two decades, many investigators have studied the bacteria attached to algae (Brock, 1966; Chan and McManus, 1969; Sieburth, 1975; Shiba and Taga, 1980). A number of alga have distinct epibacterial floras and exhibit abnormal growth if separated from them (Provasoli and Pintner, 1964; Kapraun, 1970; Fries, 1975; Bonneau 1977).

The method by which a bacterium attaches to a surface is apparently accomplished by two processes (ZoBell, 1943; Marshall, 1976). The first process is a reversible adsorption of the bacteria onto the surface. Two opposing forces seem to be important in this process: attraction by van der Waals forces and repulsion by electrical charges (Marshall et al., 1971). The second process takes place with the passage of time; that of irreversible adsorption. Different surfaces are colonized at different rates, dependent on such factors as surface tension and organic substances adsorbed to the surface (ZoBell, 1946). Rate of colonization is also dependent on the bacterial types, some of which have specialized attachment structures (Corpe, 1970).

The probability that a bacterium will encounter a surface is said to be dependent on a number of factors including motility, brownian motion (Fletcher, 1979), and hydrophobic attraction (Marshall and Cruickshank, 1973).

MATERIALS AND METHODS

Sampling Procedures

The algae utilized in this investigation were Isochrysis galbana, Monochrysis lutheri, and Pseudoisochrysis paradoxa. They are naked, unicellular flagellates which have successfully been used as food to rear oyster larvae. The samples of Isochrysis and Monochrysis were obtained from Lee Hanson's oyster hatchery in Tillamook, Oregon. Table 1 lists the identity and age of the algal samples. Pseudoisochrysis was collected from the Oregon State University Marine Science Center, Newport, Oregon. Table 2 lists the age and identity of the samples. The samples were collected in sterile 8 oz. glass jars and placed on ice until analysis, which commenced within two hours.

Microbial Enumeration and Isolation

The count of total viable, aerobic, heterotrophic bacteria was made by spreading 0.1 ml of algal sample diluted with sterile Butterfields phosphate buffer (Leininger, H.V., 1976) on plates of Difco marine agar 2216 (ZoBell, 1941; Table 3). The plates were incubated for four days at 25°C and the colony forming units (CFU) were determined from average counts of triplicate plates per dilution. For each sample, a plate containing a countable number of organisms was chosen, and every isolated colony was transferred with sterile toothpicks onto a marked spot on Difco marine agar 2216 master plates. Each plate contained 30 such marked spots.

Table 1. *Isochrysis galbana* and *Monochrysis lutheri* samples.

Culture	Description	Culture age (days)
Isochrysis galbana	air bubbled in 1 liter flask. low concentration of algae.[a]	3
Isochrysis galbana	air bubbled in 1 liter flask. high concentration of algae.	7
Monochrysis lutheri	air bubbled in 1 liter flask. low concentration of algae.	12
Monochrysis lutheri	air bubbled in 1 liter flask. high concentration of algae.	12

[a]Visual inspection

Table 2. Pseudoisochrysis paradoxa Samples.

Sample	Va[a] I.D. Code	Culture Age (days)	Culture Description[b]	Algae Count ($x10^6$/ml)
1	12	5	non-sterile[c], air bubbled, 250 ml flask.	--[d]
2	12	9	non-sterile, air bubbled, bulk culture.	--
3	1e	14	sterile[e], on shaker 250 ml flask.	--
4	12	1	non-sterile, on shaker, 250 ml flask.	--
5	12	1	non-sterile, air bubbled, bulk culture.	0.5
6	12	3	non-sterile air bubbled, bulk culture.	1.9
7	12	6	non-sterile, air bubbled bulk culture.	9.2

[a]Va stands for Virginia Institute of Marine Science

[b]Temperature of room 16-18°C

[c]Sand filtered, UV treated

[d]Not done

[e]Autoclaved

Table 3. Marine Broth 2216[a,b].

Ingredient	g/l
Peptone	5.0
Ferric citrate	0.1
Sodium chloride	19.45
Magnesium chloride	5.9
Sodium sulfate	3.24
Calcium chloride	1.8
Potassium chloride	0.55
Sodium bicarbonate	0.16
Potassium bromide	0.08
Strontium chloride	0.034
Boric acid	0.022
Sodium silicate	0.004
Sodium fluoride	0.0024
Ammonium nitrate	0.0016
Disodium phosphate	0.008

[a]Difco Laboratories, Detroit

[b]15 g Difco agar added for marine agar

Identification of Isolates

The replica plating method (Lee and Pfeifer, 1975) was used to identify most of the isolates to genus levels. The scheme used for classification is outlined in Figure 1.

Further identification utilized antibiotic resistance patterns. The antibiotics were filter sterilized (Millepore, 0.45 μm) and added to molten marine agar supplemented with 0.1% beef extract (Difco). Table 4 lists the antibiotics, their sources and the concentrations used. Cultures were replicated from supplemented marine agar plates onto the antibiotic plates and were incubated at 25°C for 48 hours.

Optimum temperature and salinity were determined for selected isolates. Marine broth was inoculated and incubated at 0, 10, 20, 37, and 50°C. Tryptone, peptone, salt media (Table 5) was used for salinity tests at the following salt concentrations: 0, 0.5, 2.0, 4.0, 6.0, and 8.0%. Salinity test plates were also incubated at 25°C.

Nutritional Requirements of Bacteria

Seven randomly selected bacterial isolates were examined for their growth factor requirements. Cultures used for inoculation of test media were initially grown in Difco marine broth 2216. The media used to grow algae at the Marine Science Center, abbreviated as salt broth (Table 6), was selected as the basal medium. Four growth factors (peptone, 0.5 g/100 ml; yeast extract, 0.1 g/100 ml; ferric citrate, 0.01 g/100 ml; and beef extract, 0.1 g/100 ml) that represented the ingredients found in marine broth 2216 supplemented with beef extract,

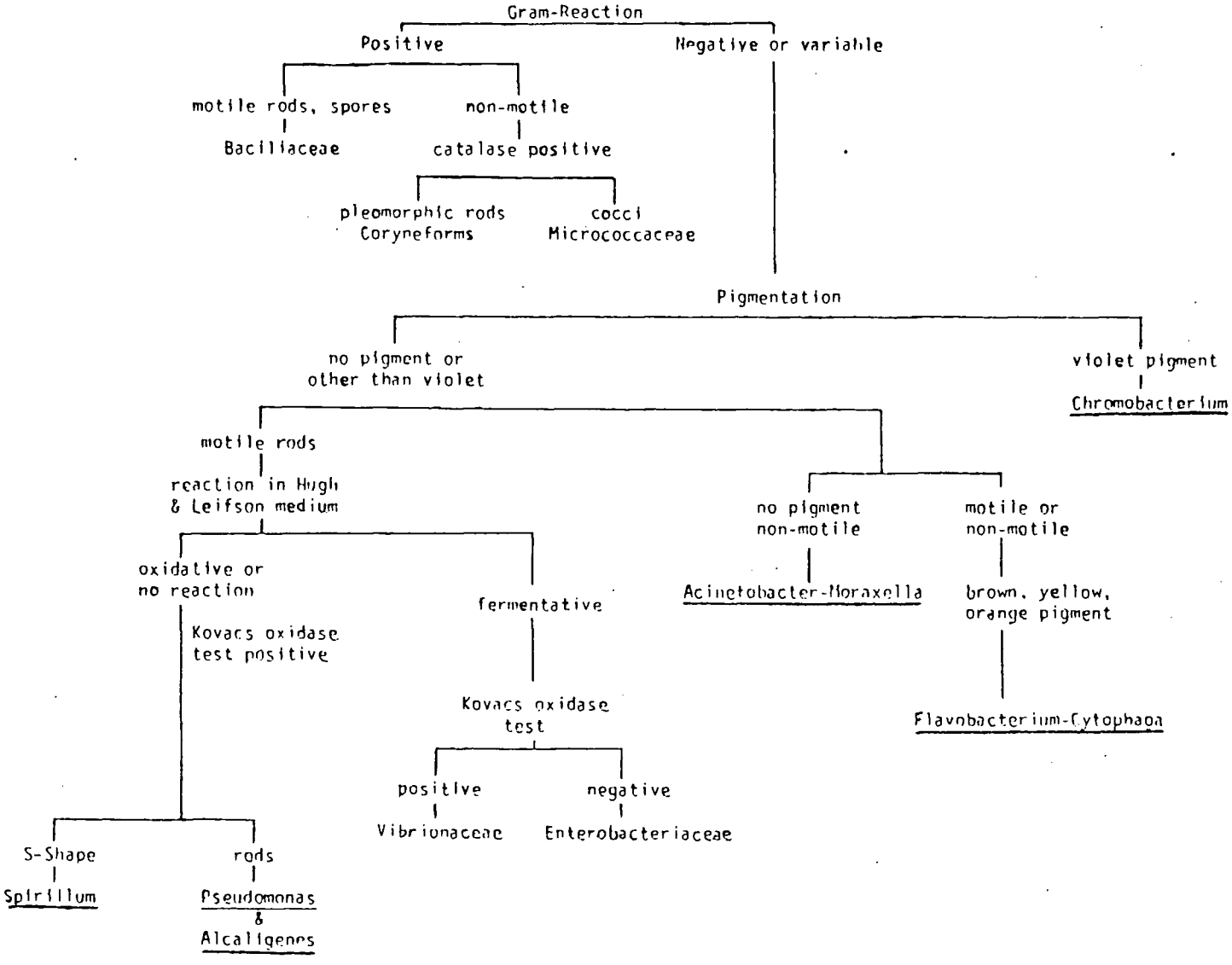


Figure 1. Classification Scheme for the Identification of the Bacterial Isolates.

Table 4. Antibiotics Used in Sensitivity Testing.

Antibiotic(s)	Symbol		Manufacturer	Concentration/l
Penicillin	Pe	Pfizerpen G	Pfizer, Inc.	80 IU
Streptomycin	Sm	Streptomycin sulfate injectable	Lilly	0.0025 g
Chloramphenicol	Cm	Chloromycetin kapseals	Parke-Davis	0.00047 g
Penicillin G and Streptomycin	Pe-Sm			40 IU + .00025 g

Table 5. Tryptone, Peptone, Salt Broth[a].

Ingredient	g/l
tryptone[b]	5.0
peptone[b]	5.0
yeast extract[b]	2.5
NaCl	5.0
glucose	1.0

[a]15g of Difco agar added for solid medium.

[b]Difco Laboratories.

Table 6. Salt Broth for Algae Culture.

Ingredient	Quantity	
Minerals		
$NaH_2PO_4 \cdot H_2O$	15 g	fill to 1600 ml with distilled water.
$NaNO_3$	225 g	autoclave 15 min at 15 psi
Iron and Trace Metals		
Ferric Sequestrene	120 g	
$CuSO_4 \cdot 5H_2O$	12 ml of 1.96 g/l	dissolve in 2000 ml distilled water,
$ZnSO_4 \cdot 7H_2O$	12 ml of 4.4 g/l	place 250 ml into 200 ml flask and
$CoCl_2 \cdot 6H_2O$	12 ml of 2.0 g/l	fill to 1600 ml with distilled water.
$MnCl_2 \cdot 4H_2O$	12 ml of 36.0 g/l	Autoclave 5 min at 5 psi
$Na_2MoO_4 \cdot 2H_2O$	12 ml of 1.26 g/l	
Vitamins		
Biotin[i]	12 mg	Dissolve in 2000 ml distilled water, place 250 ml into 2000 ml flask and fill to 1000 ml with distilled water. Autoclave 5 min at 5 psi.
Vitamin B-12	12 mg	
thiamin HCl	2400 mg	

Place 250 ml of each of the three solutions in a one liter beaker. Mix 4.5 ml with 1600 ml of sea water.

were added in various combinations to the basal media. After incubation at 25°C for four days, the growth responses were recorded as: 4+ = excellent growth; 3+ = good growth, 2+ = fair growth; and 1+ = poor growth, - = no growth.

Adhesion of Bacteria to Glass Slides

The bacteria selected for this study included three reference cultures, Moraxella sp., Staphylococcus sp., and Escherichia coli and two Leucothrix sp. isolated from the algae (type A and B). These bacteria were grown in marine broth plus 0.1% beef extract until visibly turbid. A 150 ml aliquot of supplemented marine broth was placed in six, 250 ml beakers and 0.05% Tween 80 was added to two of the beakers. Four beakers were inoculated with 1.0 ml of the above four bacterial suspensions, and two beakers containing Tween 80 were inoculated with E. coli and Leucothrix, A sp. Four sterile slides were placed in each beaker perpendicular to the surface of the broth. The beakers were then covered and incubated at 25°C. The slides were removed from beakers at various intervals, heat fixed and stained with crystal violet. The bacteria on the slides were counted with a microscope. At the same time, the number of bacteria remaining in the beaker was counted by spread plating the culture on supplemented marine agar plates.

Adherence of Bacteria to Hydrocarbons

One isolate each from the seven groups of organisms and nine reference organisms selected from our reference stock were tested as

follows. Flasks (250 ml) containing Difco marine broth 2216 plus 0.1% Difco beef extract were inoculated with each culture and incubated at 25°C until visibly turbid. A 200 ml aliquot of cell suspension was centrifuged (10,000 x G/15 min /4°C), the supernatent was discarded, and the cell pellet was resuspended in 10 ml of 0.067 M Butterfields phosphate buffer. The washing was repeated twice, and the final cell pellet was resuspended in phosphate buffer to give optical density at 400 nm of 0.3 (Bausch and Lomb, Spectronic 20). A six ml aliquot of washed, suspended cells was placed in 20 mm culture tubes in duplicate, and various volumes of test hydrocarbon (n-octane, n-pentane, n-hexane or p-xylene) were added. Following a 10 min incubation at 30°C, the contents were mixed on a mixer (Scientific Products) at maximum speed for two minutes. Upon standing at room temperature for 15 min, the hydrocarbon phase completely separated from the aqueous phase and a 2-3 ml aliquot of aqueous phase was drawn carefully with a pasteur pipet. The optical density of the aqueous phase was read at 400 nm with a Spectronic 20 Spectrophotometer (Bausch and Lomb).

Commensal Grow of Bacteria with Algae

Salt broth was used as the basal medium to test the influence of antibiotics and pH on the growth of Pseudoisochrysis to determine the kind and amount of antibiotic needed to inactivate all bacteria associated with the algae. A 250 ml of Pseudoisochrysis in salt broth obtained from the Marine Science Center was treated with various types and concentrations of antibiotics (Table 7). The effectiveness of antibiotics was measured by determining the survival of bacteria by spreading 0.1 ml

Table 7. Effectiveness of Antibiotics Against Bacteria in Algal Salt Broth.

Antibiotic(s)	Symbol	Concentration	CFU/ml
Penicillin G	Pe	45 IU 300 IU	NI[a] NI
Chloramphenicol	Cm	0.6 mg	NI
Neomycin	Nm	0.07 mg	NI
Tetracycline	Tc	0.156 mg	NI
Penicillin+ Chloramphenicol	Pe-Cm	300 IU, 0.01 g	6
Penicillin+ Chlorampenicol+ Neomycin	Pe-Cm-Nm	45 IU, 0.6 mg, 0.07 mg	
Penicillin+ Chloramphenicol+ Neomycin + Tetracycline	Pe-Cm-Nm-Tc	93.75 IU, 0.056 mg, 0.22 mg, 0.16 mg	0

[a]NI = no inhibition.

of broth onto supplemented marine agar plates and incubating at 25°C for four days.

After an effective antibiotic combination was determined, the antibiotic solution in 0.1 M phosphate buffer was added to Pseudoisochrysis culture and its effect on the growth of algae was determined.

Erlenmyer flasks containing 250 ml, each in duplicate, of salt broth, salt broth plus antibiotics and salt broth plus buffer were inoculated with twenty mls of Pseudoisochrysis (VAle, fifteen day old, on shaker, non-sterile media). The flasks were incubated at 16°C on a shaker and under a grow light. Spread plates were made from each flask at intervals to check sterility.

Growth of the algae was determined by a Coulter Counter (Coulter Electronics, Inc., Hialeah, FL).

A Coulter Electronics Size Distribution Analyzer was used to determine the relative sizes of particles present in the algal samples. Change in pH during algal growth was also monitored by a Corning 125 pH Meter.

RESULTS AND DISCUSSION

Bacteria Associated with Algae

Enumeration

The total numbers of viable, aerobic, heterotrophic bacteria associated with two cultures each of Isochrysis galbana and Monochrysis lutheri were enumerated and the counts are presented in Table 8. The bacterial numbers ranged from 5.9×10^4 to 3.4×10^5 colony forming units (CFU) per ml. The number corresponded to the visually observed density of the algae indicating, perhaps, an associated growth had occurred. The growth of bacteria on marine agar plates was slow and it took four days to form visible colonies.

Such fastidiousness is an indication that these bacteria are of the specialized types, perhaps suited for growth in association with the algae.

In a separate experiment, the number of Pseudoisochrysis paradoxa was counted during growth along with that of heterotrophic bacteria. As shown in Figure 2, the bacterial number increased as the count of algae increased. Figure 2 also shows that the number of bacteria increased at a faster rate than that of algae. As was the case with the earlier experiment, the aerobic, heterotrophic bacteria associated with Pseudoisochrysis also took four days to reach a countable size on agar plates, again indicating their fastidiousness. The bacterial numbers ranged from 7.8×10^3 to 3.9×10^6 CFU per ml, while that of algal was from 0.5 to 9.2 millions per ml.

Table 8. Microbial Load of Algal Cultures.

Culture[a]	Culture Age (days)	CFU/ml
Isochrysis galbana	3	7.7×10^4
Isochrysis galbana	7	3.2×10^5
Monochrysis lutheri	12	5.9×10^4
Monochrysis lutheri	12	3.4×10^5

[a]See Table 1 for culture description.

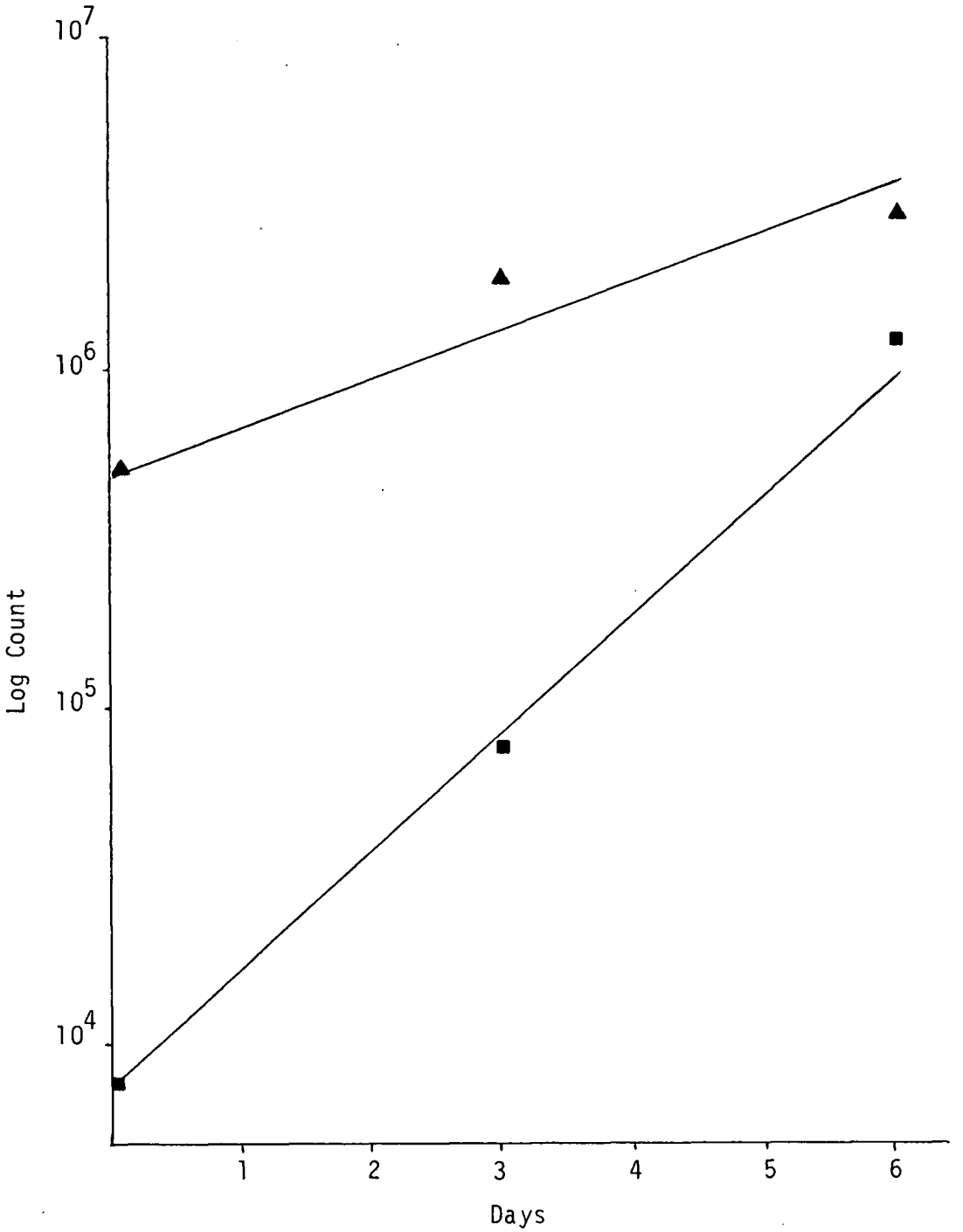


Figure 2 . Growth of Bacteria in Pseudoisochrysis Culture.
Symbols: (■) bacteria; (▲) algae.

Taxonomy

Individual colonies of bacteria associated with the samples of Pseudoisochrysis sp. were isolated and attempts were made to identify them according to the replica plating technique and the proposed classification scheme (Lee and Pfeifer, 1975). A large percentage of the organisms, however, could not be identified by this scheme. The scheme was developed for the identification of seafood associated bacteria. Apparently the microorganisms associated with the algae were sufficiently different and did not fall into a readily recognizable category.

All isolates were gram negative rods and fell into one of seven basic types as listed in Table 9.

Type A was by far the most numerous in the majority of the samples (Table 10). Reactions on replica plates by type A organisms, however, were mostly negative. Microscopic examination revealed unusual and distinct characteristics of this bacteria, that closely resembled those of Leucothrix mucor presented in Table 11. Type B was also identified as Leucothrix mucor. It differed from type A only in antibiotic resistance. Bacteria of types A and B were pleomorphic, consisting of long filaments, short filaments, rosettes or cylindrical or ovoid cells.

The morphology of Leucothrix is shown to be greatly influenced by nutritional conditions (Johnson et al., 1971; Bland et al., 1973). In marine broth, type A isolates formed short filaments, ovoid cells and rosettes, whereas in the marine broth supplemented with 0.1% beef extract, they were mainly in the form of long filaments and ovoid cells.

Table 9. Identities and Description of Bacterial Isolates.

Bacteria Type	Genus	Pigmentation	CO[a]	Catylase	Motility	Antibiotic Resistance[b]
A	Leucothrix	None	+	-	-	-
B	Leucothrix	None	+	-	-	Pe,Pe-Sm
C	Moraxella	None	+	-	-	-
D	Atypical Moraxella	None	+	-	-	Pe,Sm,Pe-Sm
E	Atypical Moraxella	None	+	+	-	Pe,Pe-Sm
F	Pseudomonas III	None	+	-	+	-
G	Flavobacterium	Orange, Yellow	-	-	+/-	Pe,Sm,Pe-Sm

[a]CO = cytochrome oxidase.

[b]See Table 3 for description and concentration of antibiotics.

Table 10. Percent Distribution of Bacteria Associated with *Pseudoisochrysis* Samples.

Sample[a]	Microbial Count (CFU/ml)	Bacteria type[b] (%)						
		A	B	C	D	E	F	G
1	1.4×10^5	68	1	1	18	0	1	0
2	1.1×10^5	41	0	7	1	0	1	16
3	3.9×10^6	13	0	5	11	0	20	9
4	5.0×10^5	1	0	1	1	0	79	6
5	7.8×10^3	2	11	0	20	28	0	0
6	7.8×10^4	57	5	24	4	0	0	0
7	3.9×10^6	57	14	0	6	0	0	0

[a]See Table 2 for description.

[b]See text for description and Table 9 for identity.

Table 11. Differential Characteristics of *Leucothrix* sp.[a].

Rosette formation: Key diagnostic characteristic
Gram -
Strict aerobes
NaCl needed for growth (0.3 - 6%)
Maximum growth temperature 30-35°C; minimum 2-5°C
Morphology long filaments of variable lengths (often longer than 100um, diameter 3-5um) composed of short cylindrical or ovoid cells, colorless, unbranched, may taper from base to apex
Gonidia (single cells)

[a]Adapted from Brock (1974).

Leucothrix sp. are common saprophytes in the marine environment (Raj, 1977). They grow as an epiphyte of microscopic marine algae and arthropods perhaps attached firmly on the surface (Pringshiem, 1957; Lewin, 1959; Brock, 1966; Anderson and Conroy, 1968; Johnson et al., 1971; Bland and Brock, 1973). Although they may survive for a short time on non-living surfaces, growth of Leucothrix sp. is stimulated when attached to living hosts (Raj, 1977).

Three types (C, D, and E) of Moraxella sp. were isolated. These were short, plump non-motile rods, cytochrome oxidase positive and were unable to ferment glucose, as determined by the modified Hugh-Leifson test (Lee and Pfeifer, 1975). Type C was sensitive to penicillin which is a typical characteristic of Moraxella, whereas the other two types (D and E) were resistant to penicillin and were classified as atypical Moraxella sp. Types D and E also differed in their catylase reactions; type D being catylase negative and type E catylase positive.

Pseudomonas type III constituted another bacterial type, type F. These were slender, polarly flagellated rods, cytochrome oxidase positive, and did not produce acid in Hugh-Leifson media.

The type G bacteria consisted of bright yellow or orange, motile or non-motile rods, and were identified as Flavobacterium sp.

The growth rates of the bacteria were measured in marine broth supplemented with beef extract and are presented in Figure 3. All of the bacteria grew at approximately the same rate, with the calculated generation times of 540 to 860 minutes. The different initial concentration was probably responsible for the different length of lag periods.

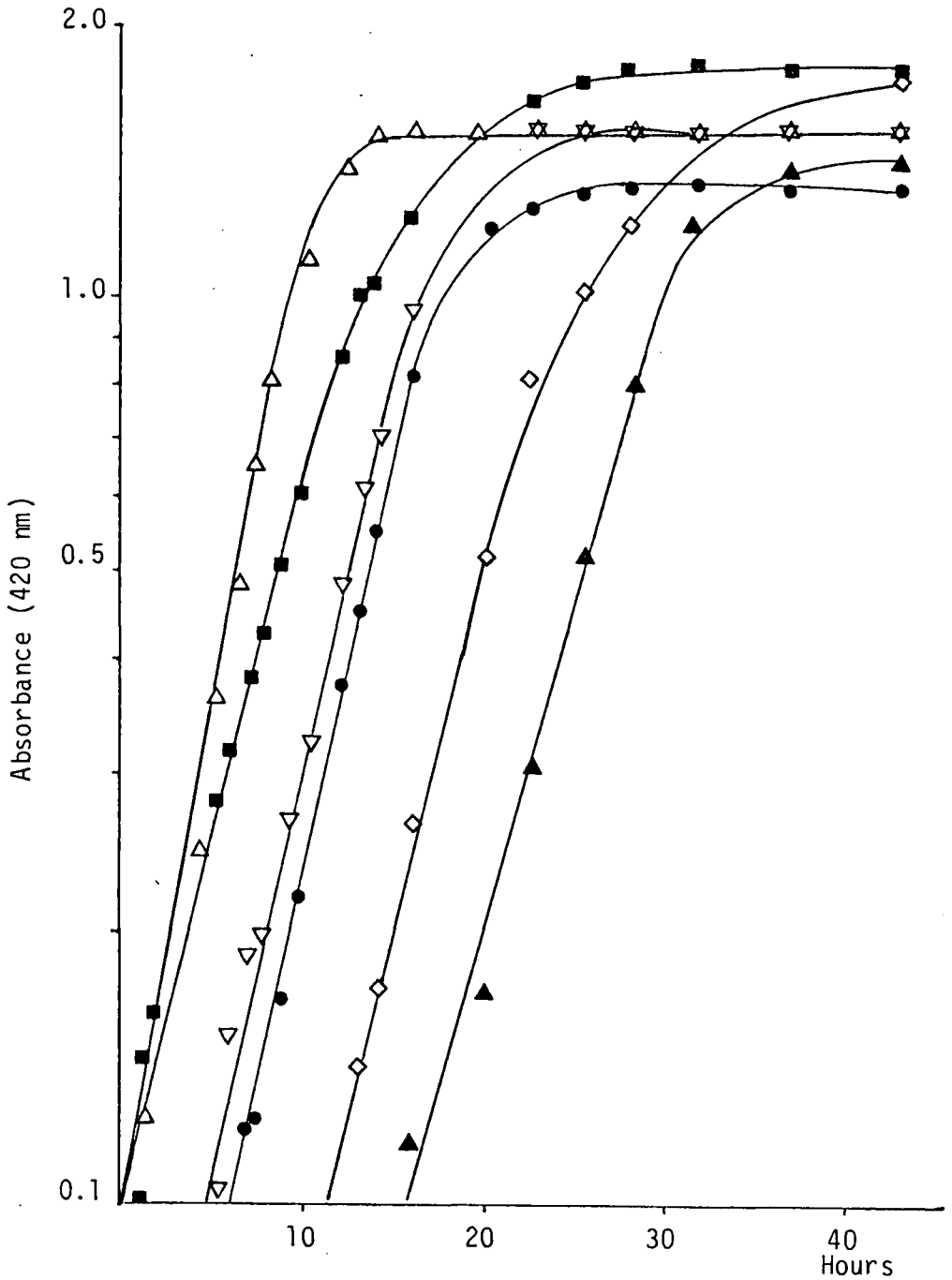


Figure 3. Growth of Bacteria in Marine Broth.

Symbols: (■) Leucothrix A sp.; (▲) Leucothrix B sp.; (●) Moraxella sp.; (◇) atypical Moraxella D sp.; (▽) atypical Moraxella E sp.; (△) Pseudomonas III sp.

Nutrient Requirements

Marine broth 2216 was especially formulated to support growth of marine bacteria (ZoBell, 1940). In view of the fastidiousness exhibited by the bacterial isolates on marine agar, it appeared that the bacteria needed additional growth factors, perhaps supplied by the algae.

To determine if the presence of algae is a requirement for the growth of bacteria, the media used to grow algae, abbreviated as salt broth, was inoculated with selected bacterial isolates. As shown in Table 6, the salt broth is similar in composition to sea water, in terms of major ions. It also contains chelating agents to maintain trace elements in solution, and organic growth factors. The salt broth, however, was not able to support growth of the bacteria (Table 12). Since all the microorganisms tested were initially isolated from algae grown in the salt broth, and the bacteria number was high, the bacteria apparently needed algae for growth. Growth of bacteria was adequate in marine broth and marine broth supplemented with beef extract had best supported a rapid and luxuriant growth.

To determine the nutrient(s) in the supplemented marine broth that stimulated bacterial growth, the salt broth was supplemented with various combinations of peptone, yeast extract, ferric citrate and beef extract. The results, presented in Table 12, show that, with a few exceptions, the bacteria grew better when ferric citrate was added.

Citrate could be one of the extracellular products of algae. Hellebust (1965) examined the organic compounds excreted by marine phytoplankton and found a large amount of the total excreted carbon in the anionic fraction. This indicated the presence of large amounts of

Table 12. Growth Factor Requirements of Selected Isolates.

Organism	Marine broth	Marine broth+B	Buffered Salt broth	Growth Factors Supplemented[a]														
				F	Y F	P F	B F	P Y F	B Y F	P B F	P Y B F	B	P B	Y B	P Y B	Y	P Y	P
Leucothrix,A	+2	+4	-	+2	+2	+2	+2	+2	+2	+2	+2	+1	-	+1	-	-	-	-
Leucothrix,A	+2	+4	-	+2	+2	+2	+2	+2	+2	+2	+2	+1	-	-	-	-	-	-
Leucothrix,A	+2	+4	-	+2	+2	+2	+2	+2	+2	+2	+2	-	-	+1	-	-	-	-
Leucothrix,A	+2	+4	-	+2	+2	+2	+2	+2	+2	+2	+2	+1	-	-	-	-	-	-
Leucothrix,B	+2	+4	-	+2	+2	+2	+2	+2	+2	+2	+2	-	-	-	+1	-	+1	-
PseudomonasIII	+2	+4	-	+2	+2	+2	+2	+2	+2	+2	+2	-	-	-	-	-	-	-
PseudomonasIII	+2	+4	-	+2	+2	+2	+2	+2	+2	+2	+2	+1	-	+1	-	-	-	-

[a]Symbols: (P) peptone 0.5g/100 ml; (Y) yeast extract 0.1g/100 ml; (F) ferric citrate 0.01g/100 ml; (B) beef extract 0.1g/100 ml, added to buffered salt broth.

organic acids or other organic anions of low molecular weight. The anionic fraction was heated with infared at reduced pressure then co-chromatographed with known organic acids, including citric acid. The heated fraction did not chromatographically correspond to any of the known acids. This unknown compound was the main excretion product of Isochrysis galbana and Monochrysis lutheri. Hellebust also found that there was considerable differences among species in both relative amount and composition of the excreted carbon material. Unfortunately, Hellebust did not include Pseudoisochrysis in his study. It appears that citrate, or some other organic acid(s) excreted by Pseudoisochrysis could play an important role in stimulating bacterial growth.

Different responses noted among bacteria to the supplements might have been due to strain or species differences. Bell, et al. (1974) reported considerable difference existing among marine bacteria in their ability to utilize algal extracellular compounds which was largely due to differences in their abilities to assimilate the compounds. Bell grew a Pseudomonas sp. and a Spirillum sp. in media containing ^{14}C-labeled algal extract. A 40-fold difference in rates of ^{14}C-label uptake was noted between Pseudomonas sp. and Spirillum sp.

Nutrients supplied by algae may or may not play an essential role in bacterial growth. Brock (1966) investigated the growth association of Leucothrix mucor and the marine algae Antithamnion sarniense. He labeled the algae with tritiated thymidine and followed the transfer of radioactivity to L. mucor by autoradiography. This investigation showed that the marine algae A. sarniense did not pass nutrients directly to the epiphytically attached bacteria, but released them into the

water. This suggested that these epiphytes may not have any preferential access to the excreted organic compounds in comparison to free living bacteria. If this is the case, then the epiphytic bacteria may need algae solely as a solid support.

Adherence of Bacteria to Solid Surfaces

The adherence of bacteria to solid surfaces was examined by counting the number of bacteria attached to a glass slide suspended in the medium. Along with Leucothrix A and B sp. isolates, Moraxella sp., Staphylococcus aureus sp. and Escherichia coli sp. from our reference culture collection were included in this study for comparison. Table 13 shows the adherence of bacteria to the slide during 48 hrs of incubation. Adherence expressed as number of bacteria per mm^2 x number of bacteria per ml^{-1} x 100 percent, ranged from the most adherent, 8.93 after 48 hrs for Leucothrix, B sp. to 0.0005 for E. coli sp. Meadows (1971) and Floodgate (1966) demonstrated that bacteria could readily attach and detach from glass slides, and that time spent on a surface was variable. Such phenomenon may account for the progressive decrease in the rate of attachment observed in this study.

In a similar experiment, Zvyagintsev (1959) found Staphylococcus sp. to be very strongly adsorbed to glass surfaces. The normal habitat of Staphylococcus is skin and mucosal membranes of warm-blooded animals. Staphylococcus sp., therefore, is expected to show greater ability to adhere to solid surfaces. The Staphylococcus sp. we studied was indeed more strongly adsorbed on the slide than either E. coli or Moraxella sp. Nevertheless, the number of Leucothrix A and B attached

Table 13. Fraction[a] of Bacteria Attached to Glass Slide.

Time (h)	Leucothrix,A	Leucothrix,B	E coli	Staphylococcus	Moraxella	Tween 80 & Type A	Tween 80 & E coli
0	0	0	0	0	0	0	0
4	0	2.38	0.01	0.11	0	0	0
24	2.05	3.0	0.001	0.50	0.08	0.07	0
48	2.75	8.93	0.0005	0.09	0.02	0.04	0.00025

[a] $\frac{\text{Number of bacteria per mm}^2}{\text{Bacteria per ml}} \times 100\%$

to the glass was 4 to 6 times greater than that of Staphylococcus sp.

A number of modes of attachment have been described, these include special attachment structures, such as fimbriae (Duguid, 1959) and holdfasts (Poindexter, 1964), and the deposition of inorganic cements (Tyler and Marshall, 1967; Mulder, 1963; Pringsheim, 1949). Most aquatic bacteria, however, have no specialized attachment structures and apparently attach by means of extracellular adhesives (Fletcher and Floodgate, 1973).

Before the bacterium can attach to a surface, it must first come close to it. Marshall and Cruickshank (1973) suggested that the hydrophobic forces were responsible for orientation of bacteria at solid liquid interfaces. They supported their hypothesis by the fact that addition of a wetting agent to bacterial suspensions completely prevented the attachment of bacteria (Marshall and Cruickshank, 1973).

We also examined the effect of a wetting agent on the attachment of bacteria. The addition of Tween 80 completely prevented adhesion of E. coli until the second day and the number of attached Leucothrix A was much less with Tween 80 than that of the control (Table 13).

Hydrophobic Behaviors of Bacteria

As hydrophobic attraction seems to play an important role in the attachment of bacteria to solid surfaces, the affinity of bacteria to hydrophobic surfaces was measured to determine the degree of attachment on solid surfaces.

A simple and semi-quanitative method to measure hydrophobicity of bacteria was developed by Rosenberg et al. (1980). They measured

adsorption of bacterial cells to various liquid hydrocarbons introduced to the culture media. This was done by spectrophotometrically measuring the absorption of an aqueous suspension of bacteria before and after addition of a hydrocarbon. The cultures which showed the greatest change in percent absorbance were considered to have the greatest affinity or attraction for hydrocarbons. The data were then presented as percent absorbance, 100% absorbance for zero adherence to 0% absorbance for 100% adhesion.

Test Hydrocarbons

The hydrocarbons tested were pentane, hexane, octane, and p-xylene. Figure 4 illustrates the adsorbance of an atypical Moraxella E sp. to various volumes of hydrocarbons. Affinity of this bacterium to p-xylene was the strongest while affinity to pentane was the least. Hexane and octane gave comparable results. The extremely low affinity for pentane was, at lease in part, due to contamination of the aqueous phase by the cell-stabilized hydrocarbon droplets, which increased the absorbance readings. An opposite effect was noted with p-xylene. The hydrocarbon of choice, therefore, appeared to be hexane or octane.

Hydrocarbon Concentration

In order to determine the optimum ration of hydrocarbon volume to that of aqueous phase for all bacteria, 0.05, 0.2, and 1.0 ml of hydrocarbon were added to 6.0 ml of bacterial suspension. In most cases, the greatest volume tested, 1.0 ml, adsorbed the maximum number of cells.

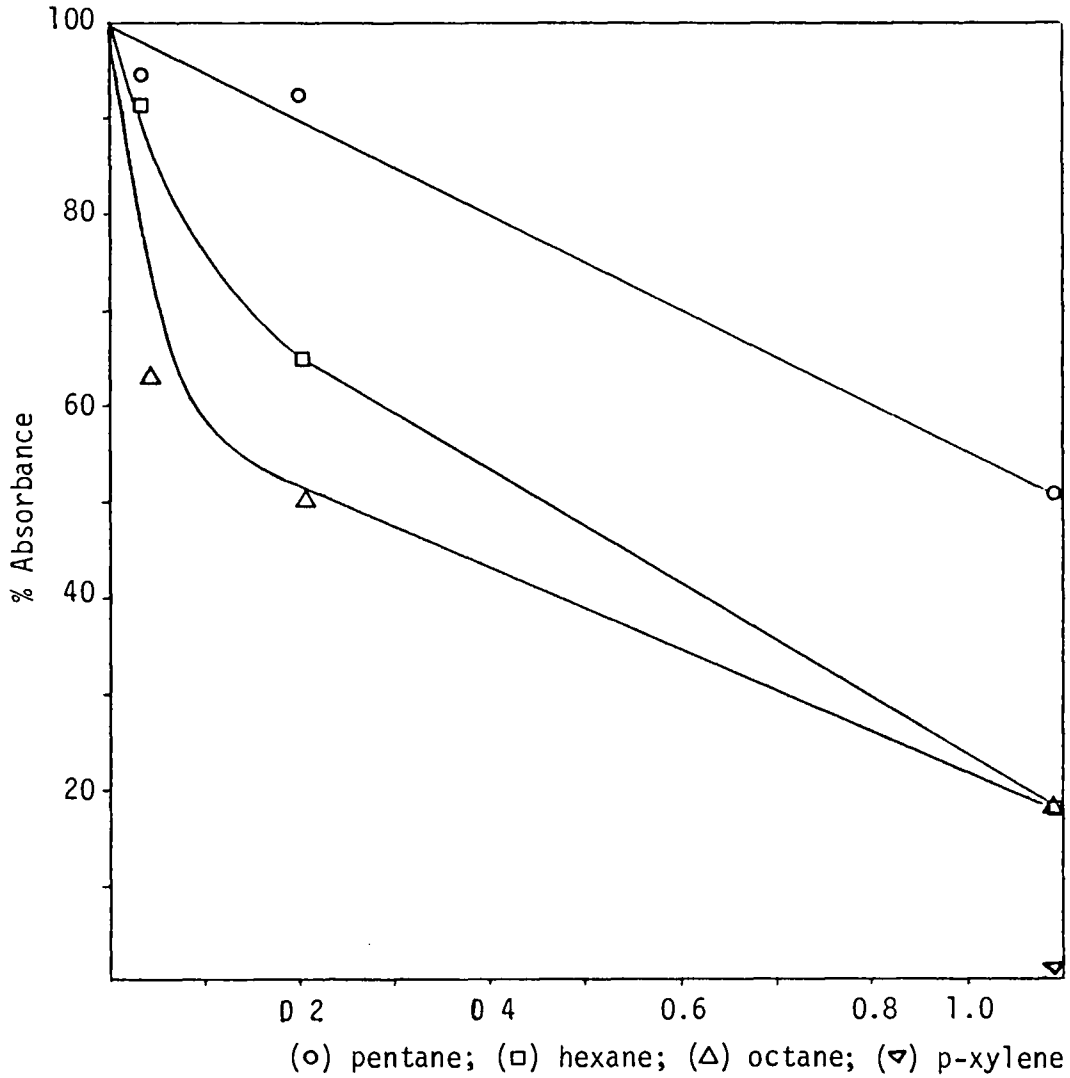


Figure 4. Affinity of Atypical Moraxella E Sp. Toward Various Hydrocarbons.

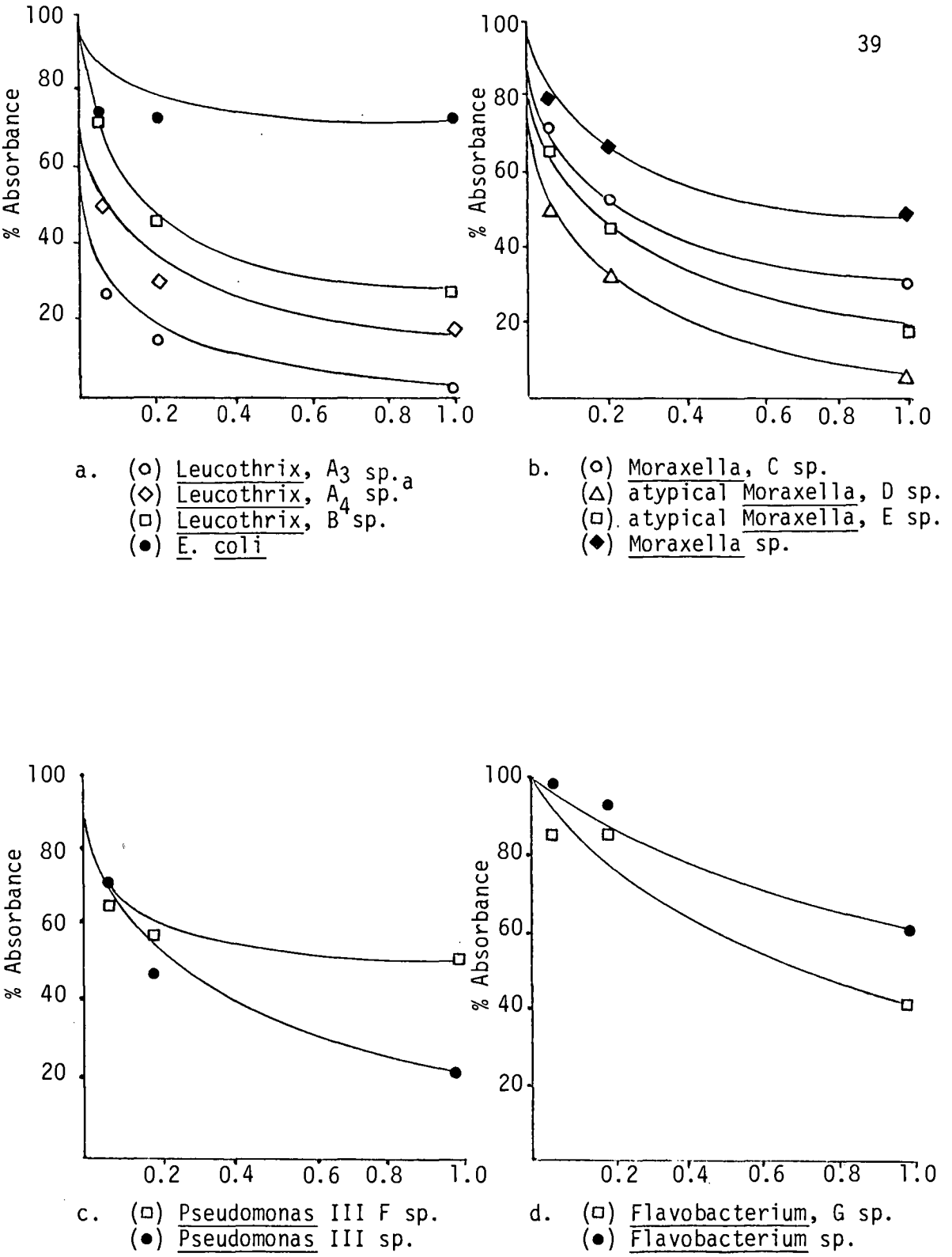
Algal Isolates

Figures 5, 6 and 7 compare the affinities of algal isolates to octane, hexane and pentane with that of seafood isolates. The affinity of bacteria measured with pentane was low and inconclusive at all three volumes tested (Figure 7). The results with hexane and octane, however, were more clear cut and data obtained with 1.0 ml of hydrocarbon per 6.0 ml of aqueous suspension gave the maximum adsorption values for all bacterial cultures tested (Figure 5 and 6).

Table 14 compares the affinity of selected algal isolates to 1.0 ml of hydrocarbons expressed as percent absorbance of the aqueous phase. With the exception of Pseudomonas III F and Flavobacterium G isolates, all algal isolates showed stronger affinity toward hydrocarbons than that of seafood isolates shown on Table 15. The zero absorbance by Leucothrix A_1, and A_2 in octane indicated complete adsorption.

Figure 5a compares the affinity of Leucothrix A and B with that of E. coli, an organism which has previously been reported to have low attraction for hydrocarbons (Kennedy et al., 1975).

Kennedy suggested that the ability to adhere to bulk hydrocarbons is a characteristic of hydrocarbon-degrading bacteria. He attributed the low attraction of E. coli toward hydrocarbons to the inability of this bacterium to degrade hydrocarbons. Rosenberg, however, found that Staphylococcus and early stationary phase cells of Serratia marcescens adhered to a variety of hydrocarbons despite their inability to degrade them. He also found that the bacteria that degrade hydro-



a. (○) Leucothrix, A_3 sp.[a]
(◇) Leucothrix, A_4 sp.
(□) Leucothrix, B sp.
(●) E. coli

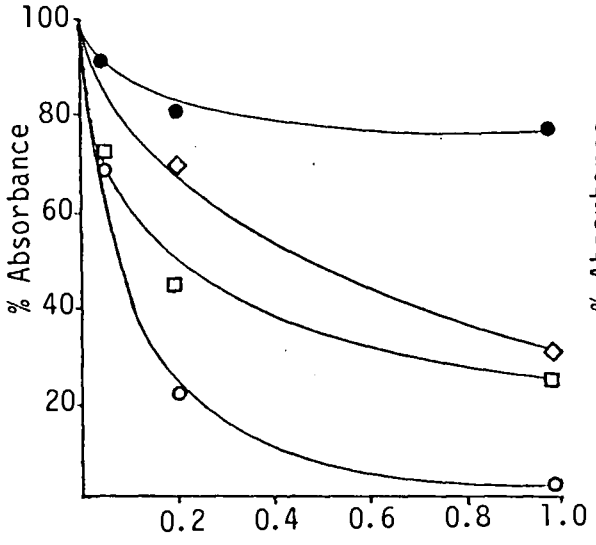
b. (○) Moraxella, C sp.
(△) atypical Moraxella, D sp.
(□) atypical Moraxella, E sp.
(◆) Moraxella sp.

c. (□) Pseudomonas III F sp.
(●) Pseudomonas III sp.

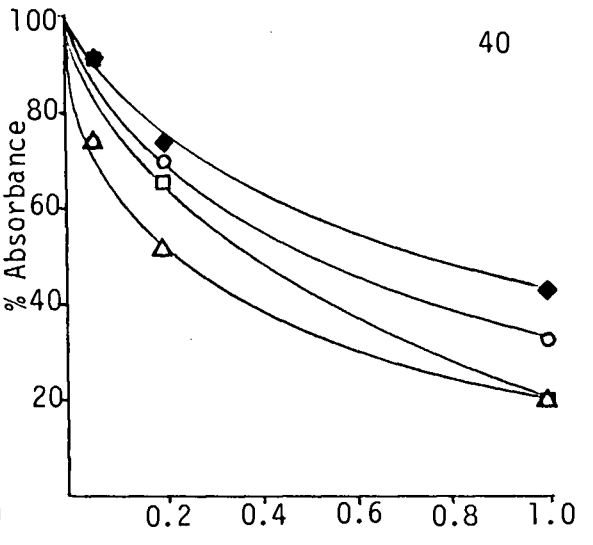
d. (□) Flavobacterium, G sp.
(●) Flavobacterium sp.

Figure 5. Affinity of Bacteria Toward Octane.

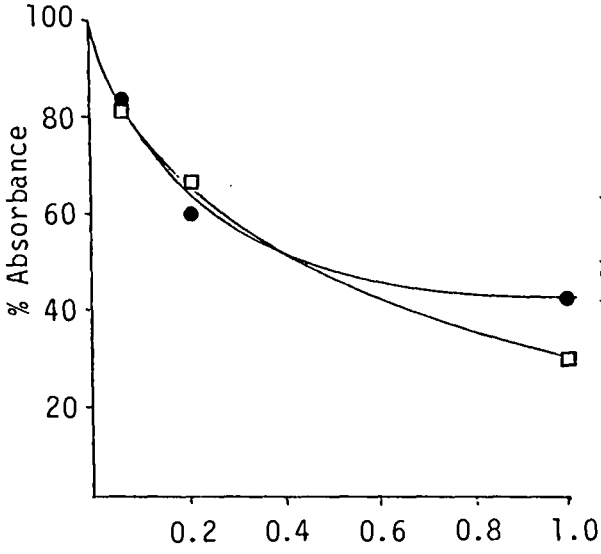
[a]Open symbols indicate algal isolates and the closed symbols are for seafood isolates.



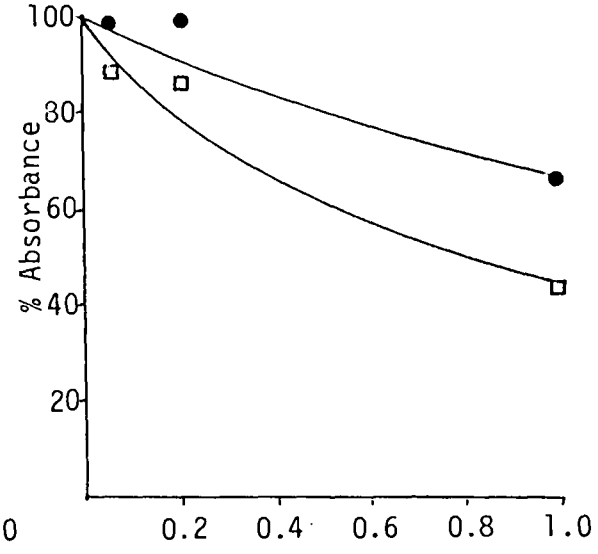
a. (o) Leucothrix, A_3 sp.[a]
(◇) Leucothrix, A_4 sp.
(□) Leucothrix, B^4 sp.
(●) E. coli



b. (o) Moraxella, C sp.
(△) atypical Moraxella, D sp.
(□) atypical Moraxella, E sp.
(◆) Moraxella sp.



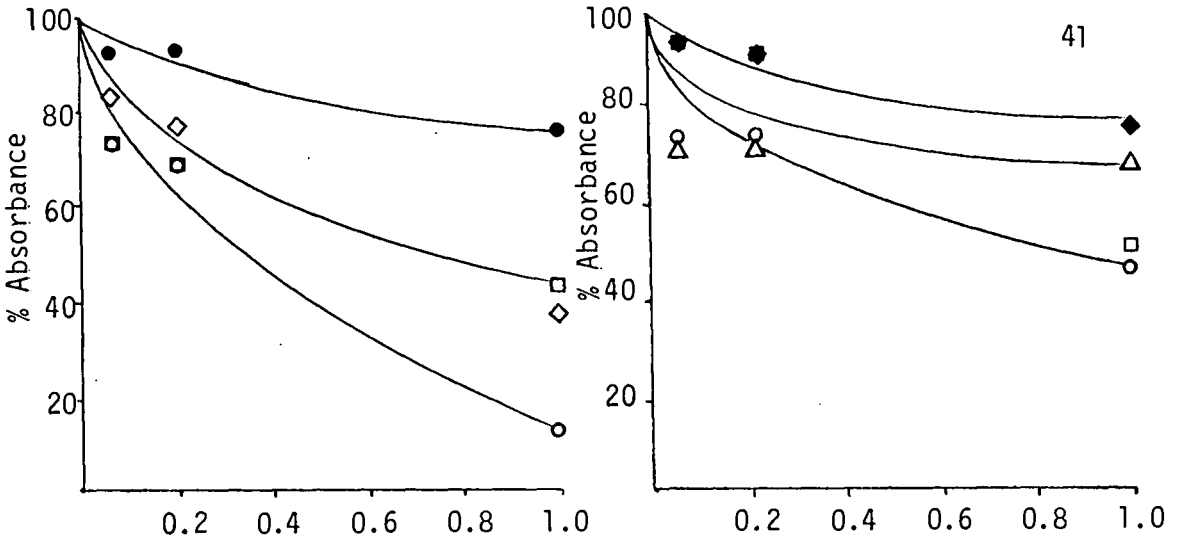
c. (□) Pseudomonas III, F sp.
(●) Pseudomonas III sp.



d. (□) Flavobacterium, G sp.
(●) Flavobacterium sp.

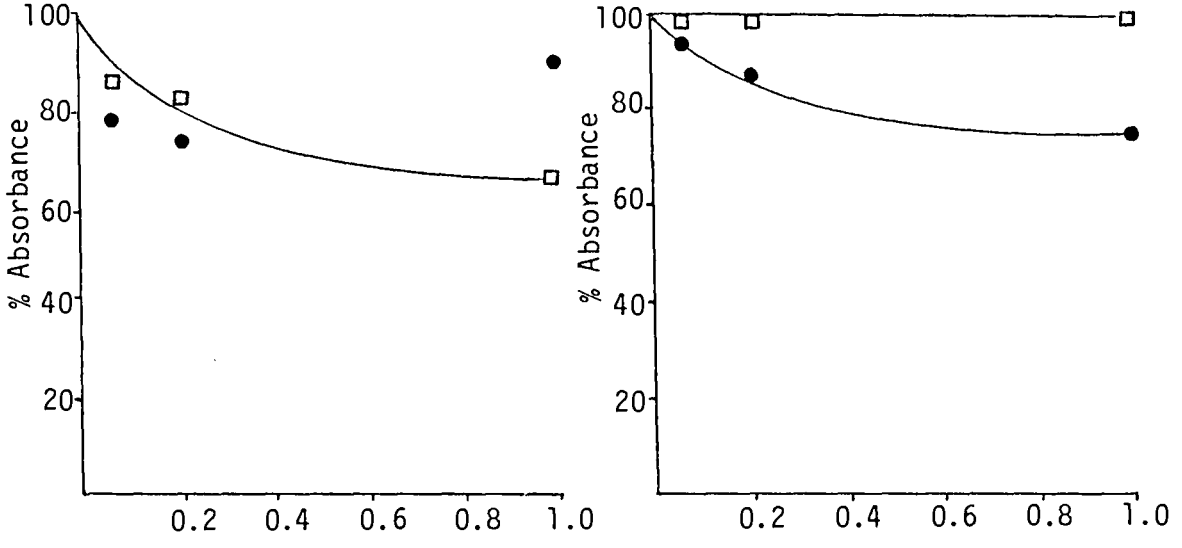
Figure 6. Affinity of Bacteria Toward Hexane.

[a]Open symbols indicate algal isolates and the closed symbols are for seafood isolates.



a. (○) Leucothrix, A_3 sp.[a]
(◇) Leucothrix, A_4 sp.
(□) Leucothrix, B sp.
(●) E. coli

b. (○) Moraxella, C sp.
(△) atypical Moraxella, D sp.
(□) atypical Moraxella, E sp.
(◆) Moraxella sp.



c. (□) Pseudomonas III, F sp.
(●) Pseudomonas III sp.

d. (□) Flavobacterium, G sp.
(●) Flavobacterium sp.

Figure 7. Affinity of Bacteria Toward Pentane (I).

[a]Open symbols indicate algal isolates and the closed symbols are for seafood isolates.

Table 14. Affinity of Bacteria Isolated from Algae Toward Hydrocarbons.

Bacteria	Percent Absorbance			
	Pentane	Hexane	Octane	P-Xylene
Leucothrix,A_1	-	-	0	0
Leucothrix,A_2	-	-	0	1.0
Leucothrix,A_3	13.0	2.0	1.8	-
Leucothrix,A_4	43.8	23.6	17.0	-
Leucothrix,B	37.8	29.0	27.2	0
Moraxella,C	47.0	32.2	27.8	1.0
Atypical Moraxella,D	68.0	21.0	5.6	-
Atypical Moraxella,E	50.2	18.0	18.0	1.0
Pseudomonas III,F	65.6	43.6	53.0	-
Flavobacterium,G	74.0	41.6	40.0	-

Table 15. Affinity of Bacteria Isolated from Seafoods Toward Hydrocarbons.

Bacteria	Percent Absorbance			
	Pentane	Hexane	Octane	P-Xylene
Arthrobacter sp.	42.4	29.0	25.6	0
Vibrio sp.	70.6	63.4	44.0	-
E. coli sp.	75.4	76.0	72.6	40.4
Moraxella sp.	75.6	40.8	48.0	20.8
Pseudomonas III sp.	88.0	27.4	24.4	0
Bacillus subtillus sp.	90.8	63.4	61.6	77.2
Cytophaga sp.	96.6	71.4	71.8	0
Staphylococcus sp.	100.0	54.0	53.0	36.6
Flavobacterium sp.	100.0	63.6	61.6	0

carbons did not adsorb more strongly to the specific hydrocarbons they degrade. We did not test the hydrocarbon degrading ability of our isolates.

Some of the algal isolates adsorbed more poorly to hydrocarbons than those of the related seafood isolates. As an example, a seafood isolate, Pseudomonas III sp. exhibited a fairly strong attraction for octane (24.4% absorbance) as compared to that of an algal isolate Pseudomonas III sp. (53.0%); (Tables 14 and 15). This could suggest that some of the algal isolates might have been transients that did not grow in association with algae or that not all species in the same genus share a characteristic affinity for hydrocarbons. It is not even clear whether the affinity of bacterium to hydrocarbon is a stable or a transient characteristic. Rosenberg et al. (1980) has noted an increase in hydrophobility with increase in age of Serratia marcescens. They also found that the rough mutant E. coli J-5 had a greater affinity to hydrocarbons than E. coli K-12. Most algal isolates, however, showed greater affinity to hydrocarbons than their seafood counterparts.

Figure 5b illustrates the similarities and difference between algal isolates Moraxella C sp., atypical Moraxella D and E sp. and seafood isolate Moraxella sp. The seafood isolate exhibited the low affinity for octane of 48.0%, whereas atypical Moraxella D and E sp. showed the stronger affinity of 5.6 and 18.0%, respectively.

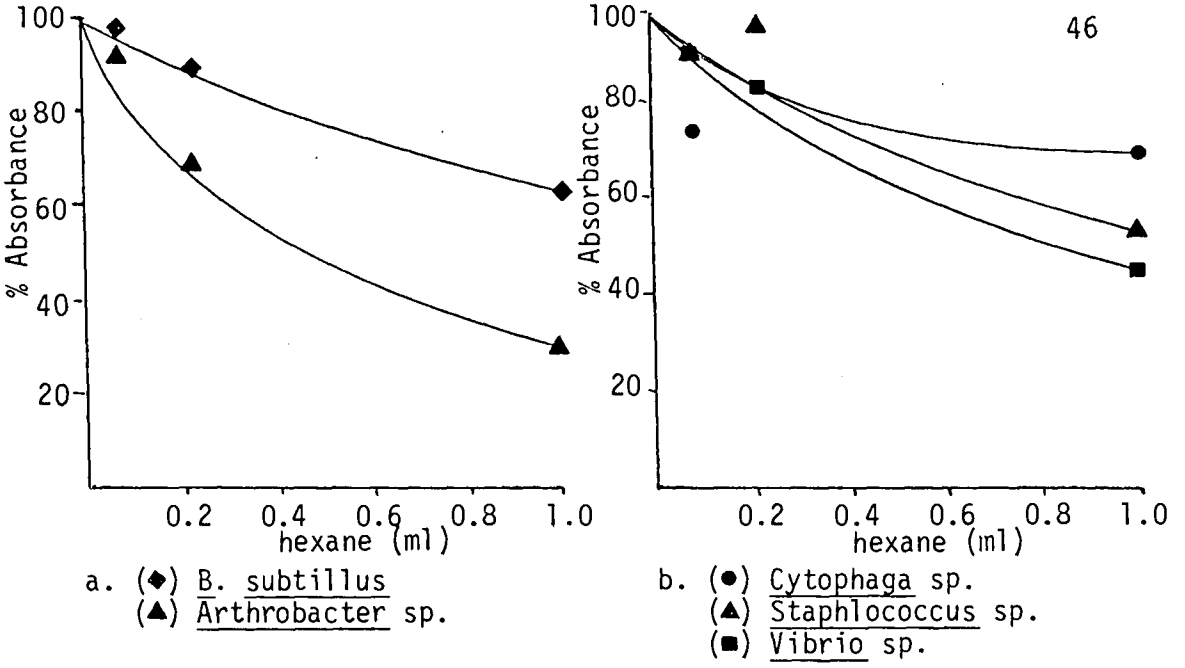
A Flavobacterium sp. isolate from the algae showed a slightly higher attraction for octane (40.0%) than that from the seafood (61.6%), but neither may be considered having strong affinity to hydrocarbon (Figure 5d).

Seafood Isolates

The strong affinity toward hydrocarbon exhibited by *Arthrobacter* sp. is of some interest (Figure 8 and 9). *Arthrobacter* sp. are common soil microorganisms usually associated with mud, and they constituted the majority of bacteria associated with shrimp reared in an earthen pond (Vanderzant *et al.*, 1971). Its strong affinity to hydrocarbon and perhaps to solid surfaces may explain the difficulty encountered in cleaning such shrimp. Another microorganism that infest fish holds and are difficult to eliminate is *Moraxella* sp. (Lee and Pfeifer, 1977). The low affinity of this bacterium to hydrocarbon may indicate a constant resupply due to rapid growth, rather than by adsorption to solid surfaces, as a cause for this difficulty.

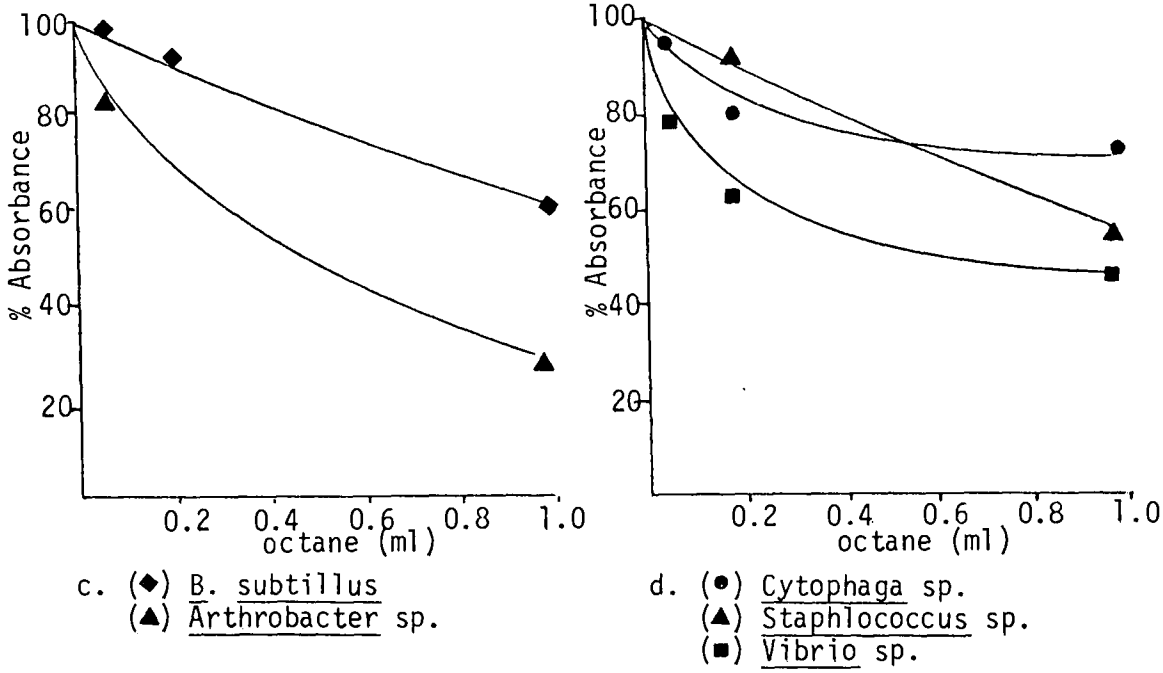
Affinity to Hydrocarbon vs Adhesion to Solid Surface

As previously discussed, hydrophobic attraction is thought to play an important role in the orientation of bacteria to solid surfaces. If this is true, then bacteria which have a strong affinity for hydrocarbons would attach to surfaces at a greater rate. This hypothesis is supported by our finding that *Leucothrix* sp. was strongly adsorbed to glass surfaces and also showed a high affinity for hydrocarbons. More than fifty percent (51%) of all bacteria present on *Pseudoisochrysis* sp. were *Leucothrix* sp. On the other hand, *E. coli* showed very little attachment to glass and, likewise, showed a very low affinity for hydrocarbons.



a. (◆) B. subtillus
(▲) Arthrobacter sp.

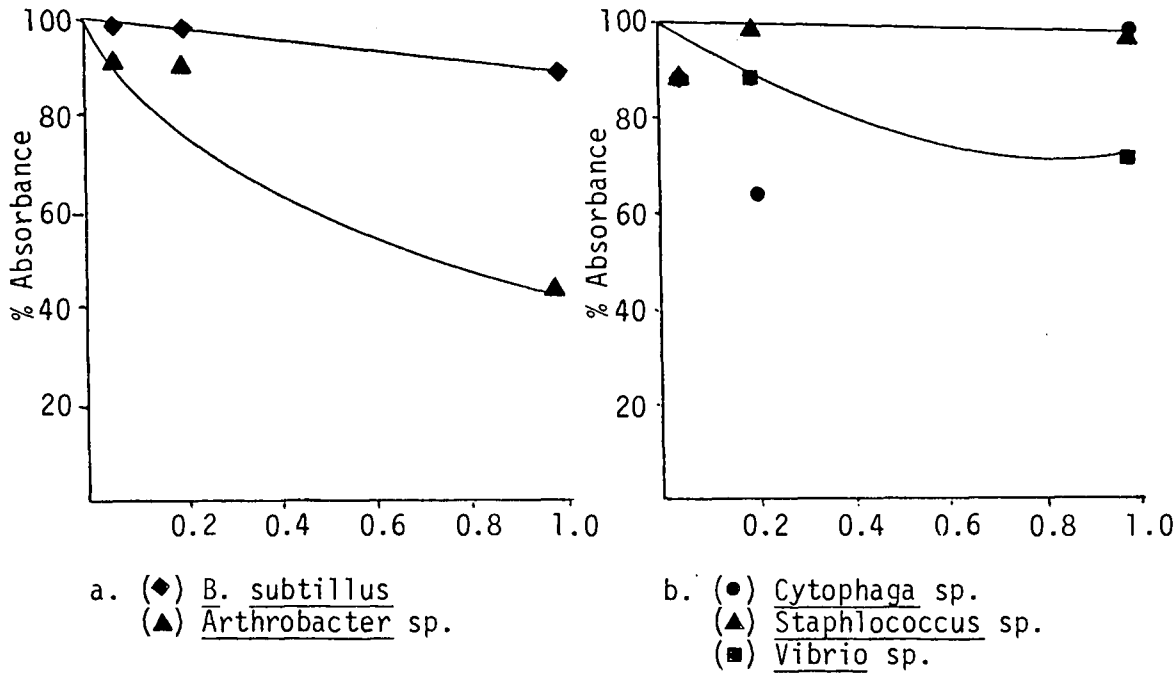
b. (●) Cytophaga sp.
(▲) Staphlococcus sp.
(■) Vibrio sp.



c. (◆) B. subtillus
(▲) Arthrobacter sp.

d. (●) Cytophaga sp.
(▲) Staphlococcus sp.
(■) Vibrio sp.

Figure 8. Affinity of Bacteria Toward Hexane and Octane.



a. (◆) B. subtillus
(▲) Arthrobacter sp.

b. (●) Cytophaga sp.
(▲) Staphlococcus sp.
(■) Vibrio sp.

Figure 9. Affinity of Bacteria Toward Pentane (II).

An exception was found with Staphylococcus sp., which adhered strongly to glass slides but displayed only a moderate attraction for hydrocarbons. It appears, however, a large majority of bacteria we isolated from algal cultures were those firmly attached to the algae.

Commensal Growth of Bacteria and Algae

To determine the role of bacteria on the rate of algal growth, algal inoculum was treated with a mixture of antibiotics and growth of subsequent axenic algae was compared to that of untreated. We determined that 93.75 IU of penicillin G, 0.056 mg of chlorompherical, 0.22 mg of neomycin and 0.16 mg of tetracycline per ml was effective in eliminating bacteria. The growth of algae, determined by a Coulter counter, is presented in Figure 10. The number of algae in the antibiotic treated media did not increase, while the algae in the untreated media increased at the rate of 24.0×10^3 cells per ml per hr. Thus, it appeared that the presence of bacteria was necessary for algal growth.

Kain and Fogg (1958) reached similar conclusions in their experiments with the algae Asterionella japonica. Johnston (1963) found bacteria-free Skeletonema costatum grew poorly in seawater enriched with nitrate, phosphate, silicate, and chelated trace metals, but it grew distinctly better in the same media if bacteria were present. Johnston attributed this effect largely to production of vitamin B_{12} by the bacteria.

The pH of algal cultures sometimes change drastically. To make sure that the antibiotic would not cause a shift in pH, the broth was

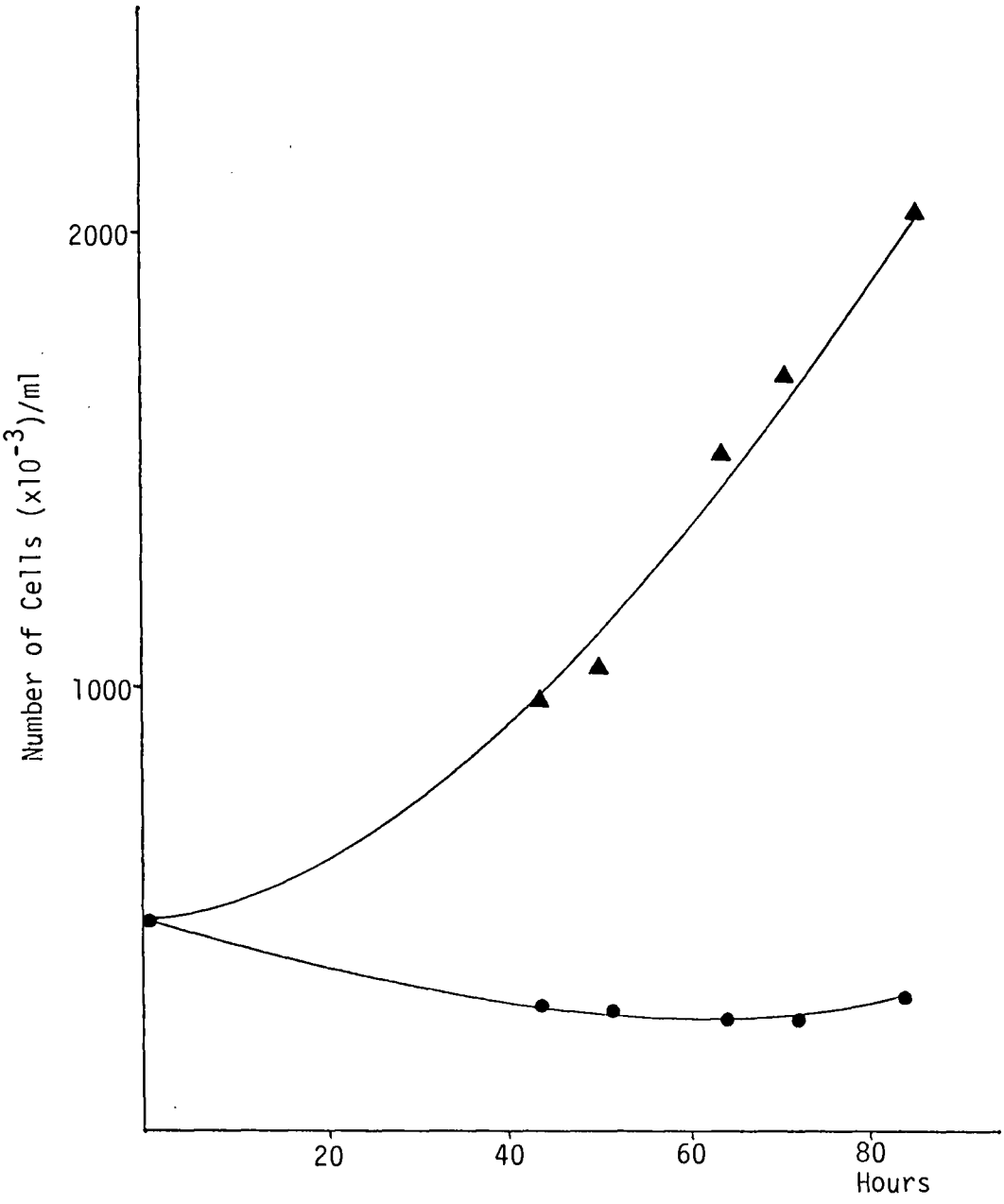


Figure 10. Influence of Antibiotics on the Growth of Pseudoisochrysis sp.

Symbols: (▲) salt broth;
(●) antibiotic treated salt broth

prepared with phosphate buffer and the pH was monitored throughout in buffered media. As shown in Table 16, the pH in the salt broth increased to 8.72 whereas that of the antibiotic treated media leveled off at 5.67. The buffered salt broth stabilized at a pH near 7.0. The algae, however, exhibited the same rate of growth (24.0×10^3 cells per ml per hr), both in buffered and unbuffered salt broth. A pH between 5 and 8 was previously shown to have no effect on algal growth (Small, personal communications). The reduced algal growth in antibiotic treated medium, therefore, appeared to be due to other factor(s).

The fact that the numbers of algae decreased in the antibiotic treated media, instead of remaining constant, or showing a slow rate of increase was puzzling. Therefore, we attempted to examine the particle size distribution in both antibiotic treated and untreated cultures. Figure 11 shows the particles found in untreated cultures were relatively uniform in size. No similar peak was detected from the antibiotic-treated culture and the peak found represented much smaller particles. Small (personal communication, 1981) recently communicated his experience of using antibiotics to eliminate bacteria from algal cultures and related that he could not find a low enough antibiotic concentration which would kill the bacteria but would not harm the algae. If antibiotics were used in high concentrations, he found that the algae were "literally blown apart". The small particles we observed could have been the debris of broken algal cells and, if so, we could not demonstrate that the algae had failed to grow in the absence of bacteria.

Axenic culture of algae is difficult to obtain. Part of the reason undoubtedly is due to strong affinity of bacteria to the algal cells.

Table 16. pH Change in Algal Culture.

Time (hr)	Salt Broth	Antibiotic Treated Salt Broth	Buffered Salt Broth
0	7.49	5.37	6.40
44½	8.28	5.13	7.08
64½	8.70	5.67	7.07
86¼	8.72	5.67	6.93

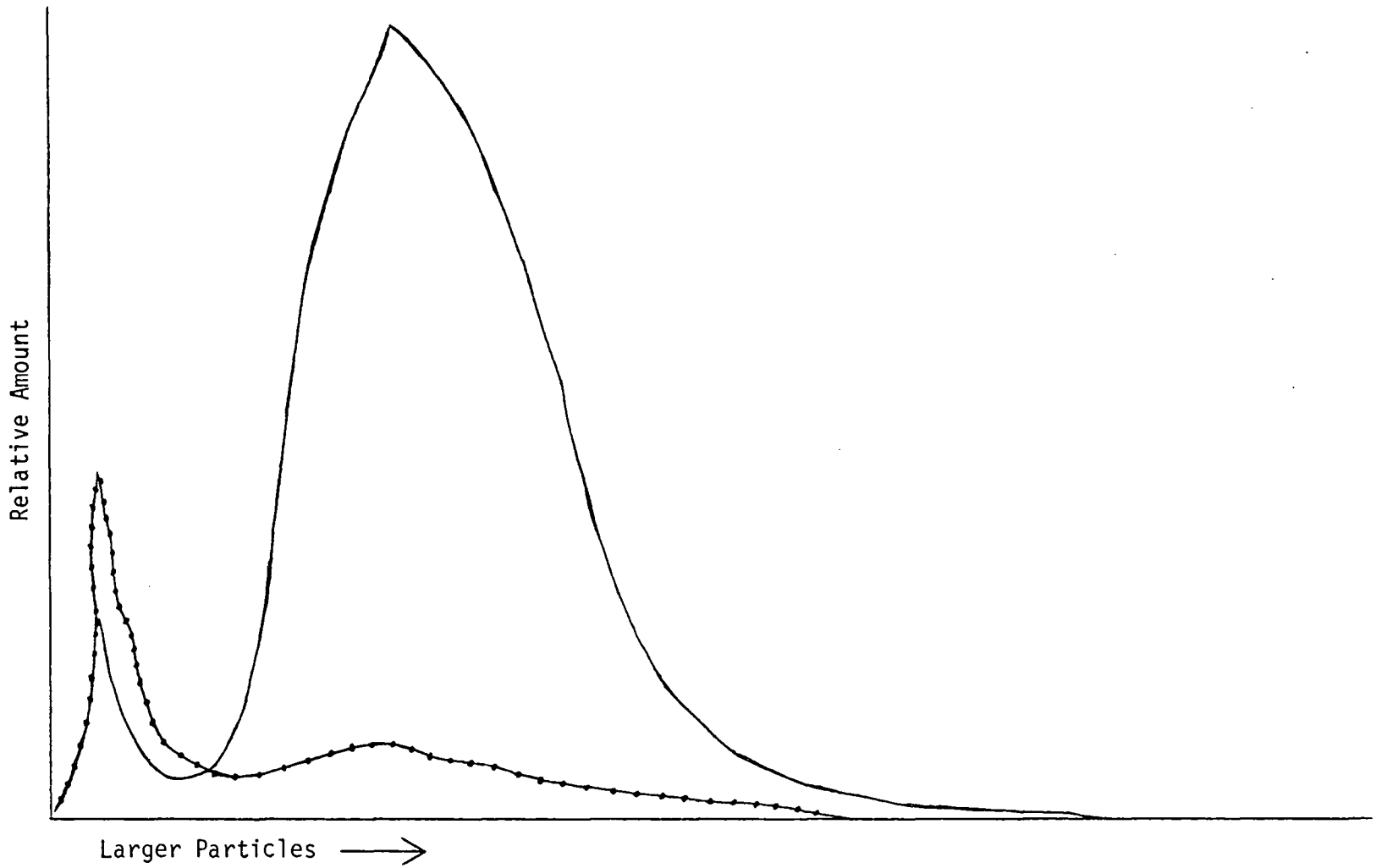


Figure 11. Particle Size Distribution in Algal Cultures.
Symbols: (•—•) antibiotic treated culture; (—) untreated culture.

SUMMARY

Aerobic, heterotrophic bacteria associated with oyster larvae feed algae Isochrysis galbana, Monochrysis lutheri and Pseudoisochrysis paradoxa were isolated and enumerated. The bacteria associated with Pseudoisochrysis sp. were identified and their growth requirements and kenetics were determined. The abilities of these isolates to attach on solid surfaces and hydrophobicity were measured to assess their roles in commensial growth with algae. The results and conclusions are summarized below:

1. Bacterial numbers from Isochrysis galbana and Monochrysis cultures ranged from 5.9×10^4 to 3.4×10^5 CFU per ml. Bacterial numbers from Pseudoisochrysis paradoxa cultures ranged from 7.8×10^3 to 3.9×10^6 CFU per ml.
2. The majority of bacteria associated with Pseudoisochrysis sp. belonged to genus Leucothrix (51%). Also present were mem bers of Pseudomonas III sp. (19.1%), atypical Moraxella sp. (16.8%), Moraxella sp. (7.2%) and Flavobacterium sp. (5.9%).
3. Growth of algal bacteria on marine agar was fastidious and took four days to form visible colonies. Supplementation of marine agar with 0.1% beef extract stimulated bacterial growth.
4. The bacteria did not grow in buffered salt broth in which the algae had been grown unless supplemented with 3×10^{-4} M ferric citrate.
5. Most algal bacterial isolates adsorbed strongly to solid surfaces and also exhibited strong affinities to hydrocarbons.

Leucothrix sp. was most strongly adsorbed to glass surfaces (0% absorbance) and also showed a high affinity for hydrocarbons (2.75 bacteria per mm^2 x number of bacteria per ml^{-1}x100 percent).

6. Pseudoisochrysis paradoxa failed to grow in an antibiotic treated medium. This, however, appeared to be due to the detrimental effect of the antibiotics rather than due to the removal of the bacteria.

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