STUDIES ON SCREENING OF ANTIBACTERIAL SUBSTANCES AGAINST SHRIMP PATHOGENIC *Vibrio sp.*

DISSERTATION SUBMITTED BY

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DEDICATED TO MY FATHER

CERTIFICATE

Certified that the dissertation entitled, "**Studies on screening of antibacterial substances against shrimp pathogenic Vibrio sp.**" is a bonafide record of work done by Kum. **A. Priyalekshmi** under our guidance at the Central Marine Fisheries Research Institute during the tenure of her M.Sc (Mariculture) Programme of 1994-96 and that it has not previously formed the basis for the award of any other degree, diploma or other similar titles or for any publication.

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I hereby declare that this thesis entitled, **"Studies on screening of antibacterial substances against shrimp pathogenic *Vibrio sp.*"** is based on my own research and has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar titles or recognition.

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सारांश

वर्तमान अनुसंधान में चिंगट रोगजनक वी. एल्गिनोलिटिकस में कई एन्टिबयोटिक, एन्टिसेप्टिक और प्राकृतिक पदार्थों का संदमन करने पर होनेवाले प्रभाव पर अध्ययन किया गया.

परीक्षण किए गए 22 एन्टिबयोटिकों में विब्रियो एल्गिनोलिटिकस 10 के प्रति संवेदी और बाकी के प्रति प्रतिरोधी देखा गया.

संवेदी एन्टिबयोटिकों में क्लोराम्फीनिकोल, को–ट्राइमाक्सज़ाइन(3.0 एम सी जी) अधिक प्रभावकारी देखे गये.

ऑगमेंटिन (10 एम सी जी), अमोक्सिसिलिन(10 यू जी), स्ट्रेप्टोमाइसिन (10 एम सी जी), जेन्टामाइसिन (10 यू जी), कानामाइसिन (30 यू जी), एरिथ्रोमाइसिन (15 यू जी), नोरफलोक्सासिन (10 एम सी जी), क्लिन्डामाइसिन (2 एम सी जी) जैसे एन्टिबयोटिक रोगजनक के प्रति संवेदक हैं.

वी. एल्गिनोलिटिकस बाकी सभी एन्टिबयोटिकों जैसे सेफालोराइडिन (30 एम सी जी), लिनोमाइसिन (2 एम सी जी), मेतिसिलिन (5 एम सी जी), ओलियन्डोमाइसिन (15 एम सी जी), पेनसिलिन (10 यूनिट), टोब्रामाइसिन (10 एम सी जी), टेरासाइक्लिन (30 एम सी जी), ऑक्सीटेट्रासाइक्लिन (30 यू जी), टियामुलिन (30 यू जी), बासिट्रासिन (10 यू जी) के प्रति प्रतिरोधी था.

दस एन्टिसेप्टिक रसायनों में 6 अवरुद्ध रोगजनक वी.एल्गिनोलिटिकस में प्रभावकारी देखा गया.

कोपर सलफेट (0.5 %), फोरमलिन, एक्रिफ्लेविन (50 पी पी एम), मेथिलीन ब्लू (10 पी पी एम) अधिक प्रभावकारी और पोटासियम डाइ क्रोमेट(1.0%), मालकाइट ग्रीन (500 पी पी एम) कम प्रभावकारी देखे गए.

विरकोन नामक एक नया उत्पाद, जो रोगाणुनाशक है, 0.5 % स्तर में प्रभावकारी देखा गया. यह मिश्रित अनछना सूप के संवर्धन में वी. एल्गिनोलिटिकस की वृद्धि पर्याप्त मात्रा में कम करता है.

प्राकृतिक उत्पादों (बयोएक्टीव कोम्पाउन्ड्स) के परिणामों से यह ज्ञात हुआ कि वी. एल्गिनोलिटिकस के नियंत्रण के लिए स्पंज का सार उपयुक्त करना अच्छा होगा.

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PREFACE

The earliest record of aquaculture occurred in China, where artificial hatching and rearing of carp (*Cyprinus carpio*) dated back to 2000 BC. Since that time, man has extensively cultured species such as the milkfish (*Chanos chanos*), tilapia (*Oreochromis mossambica*), yellow tail (*Seriola quinquiradiata*), rainbow trout, Atlantic salmon (*Salmo salar*) and many other salmonid species. Aquaculture was first carried out in Europe by the Romans with oysters and now the culture of shellfish such as clams, oysters and prawns is common. All the culturable species are subjected to infectious diseases and their impact on the economic viability of the aquaculture industry has become increasingly important. According to Meyer (1991), **disease problems constitute the largest single cause of economic losses in aquaculture'.** Now as the natural fisheries provided by the open seas have declined and the world population has grown, aquaculture products are in great demand.

Global aquaculture production is estimated at nearly 10 million metric tonnes annually and contributes over 12% of

the total consumed fish and shellfish. The industry is now projected to grow at an annual rate of 8% through the year 2000 (FAO). This industry growth will only come from increased cultural efficiency in existing facilities, because there will be restrictions on water use, and increased production from increased acreage will be unlikely. One of the primary means for increasing cultural efficiency will be the improvement of animal health and the control of infectious diseases.

Disease is one of the main factors limiting the survival, growth and production of farmed fish and shellfish. It has been estimated that about 10% of all cultured aquatic animals are lost as a result of infectious diseases. Highly pathogenic viruses and bacteria can cause mortality even over 90% of hatchery-reared populations.

Poor husbandry, over crowding, unsuitable water quality and dietary imbalances are the major factors for the occurrence of diseases in shrimp culture systems. Diseases can be of infectious and non-infectious aetiologies. Infectious diseases are caused by viruses, bacteria, fungi and other parasitic organisms. Common virus diseases encountered in shrimp culture systems are: Infectious Hypodermal and

Hematopoietic Necrosis Virus (IHHNV), Hepatopancreatic Parvo like Virus (HPV), Lymphoid Organ Parvo like Virus (LOPV), Lymphoid Organ Vacuolization Virus (LOVV), Baculovirus Penaei (BP), Monodon Baculo Virus (MBV), Baculo viral Midgut gland Necrosis Virus (BMN), and Type - C Baculo Virus (TCBC). The most important fungal disease in larvae (larval mycosis) are caused by *Lagenidium* and *Fusarium spp.*

Bacterial diseases in shrimp culture are caused by several species of bacteria such as *Vibrio*, *Beneckia*, *Leucothrix* *Pseudomonas* and *Aeromonas*. In addition, rickettsial diseases are also reported.

Vibrio sp. establish lethal infections along with other stress factors such as deteriorated environment, nutritional imbalances and predisposing lesions. In addition to mortality caused by bacteria, the bacterial infections cause growth retardations and several other complications like black discolouration of shrimp carapace and erosion of the appendages and the telson (Lipton, 1994). These imperfections can reduce the sale value of the shrimp.

It is possible that antibiotic and antiseptic chemical treatments will help to overcome the infections. However, the

causative stress factors are to be eliminated. If the stress is not alleviated, the shrimps will not respond to the treatment. It has been observed that individual shrimp with septicemic conditions collected from a single pond could be infected with more than one bacterial species and that different animals are infected with different species of bacteria. In order to carry out antibiotic treatment for such multiple infections, it is essential to conduct sensitivity tests with each of the isolate in order to find an effective antibiotic for all pathogenic isolates. Studies on screening of antibiotics, antiseptic compounds and natural compounds are to be undertaken and then only advocated for use in the hatchery and culture systems. Considerting the importance of bacterial disease management in the growing shrimp culture activities, the present research work was undertaken with the following objectives:

1. To screen suitable antibiotic compounds,
2. To screen effective antiseptic compounds,
3. To screen some natural (bioactive) substances against shrimp pathogenic *Vibrio sp.*,

   and
4. To test the suitable conditions required for their maximum activity.

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INTRODUCTION

Being the most important species of cultured shrimp, *Penaeus monodon* has been a frequent subject of disease investigations by pathologists. Naturally distributed throughout the Indo-West Pacific region from 30^{o} E to 155^{o} E longitude and from 35^{o} N to 35^{o} S latitude, *P. monodon* is most abundant in the tropical waters of Indonesia, Malaysia and the Philippines. It has become an important culture species in countries within this range, especially, Indonesia, Thailand, India, the Philippines, Vietnam and Taiwan. *P. monodon* is considered to be exceptionally hardy, but increasing culture densities and environmental degradation have contributed to disease problems. Serious diseases of viral, bacterial, fungal, Protozoan, rickettsial and of unknown aetiologies have been reported.

Ruangpan (1982) reported that vibriosis affected protozoeal stages and caused heavy mortalities (upto 80%) in *P. monodon* hatcheries. Anderson et al., (1988) reported several incidence of 'vibriosis' in juvenile *P. monodon* cultured in Malaysian brackish water ponds and isolated bacteria belonging to the genus *Vibrio* and *Pseudomonas* and other Gram negative bacteria. They noted that 'vibriosis' in juvenile

shrimp could be treated successfully by adding antibiotics to the diet; for the semi -intensive ponds studied, however, chemotherapy was not an economical option. Mortalities were reduced after the farmers began to remove excess detritus from dried ponds followed by application of Calcium oxide at a rate of 0.5 kg/m2.

Influence of *Vibrio* pathogen on shrimps:

Perhaps as a consequence of the large number of *Vibrios* in the normal shrimp microflora, opportunistic *Vibrio spp.* have been the most common bacterial pathogens of cultured shrimp. *Vibrio spp.* appeared to establish lethal infections following primary infections with other pathogens, environmental stress, nutritional imbalance and/or predisposing lesions (Lightner, 1988). According to Lightner et al.,(1992), some more recently occurring disease syndromes of penaeid shrimp were caused by *Vibrio spp.* which were behaving more like true pathogens than opportunistic invaders. Involvement of other Gram negative bacteria and Cytophaga - type filamentous bacteria in serious disease outbreaks of cultured shrimp in Asia were rarely documented.

Vibriosis caused by species of the genus *Vibrio*, has been described as the most serious disease of Penaeid shrimps

(Egidius, 1987). The loss in production of *Penaeus japonicus* due to vibriosis has been estimated at about 30.8 metric tonnes/yr in Japan (Sano and Fukudo, 1987). Increased *Vibrio* populations in larval rearing tanks water was one of the factors that reduced the survival rate of larvae and post - larvae of *Penaeus indicus* (Hameed, 1994). Furthermore, Yasuda and Kitao (1980) observed poor growth of the shrimp when *Vibrio* populations were dominant in the gut. Of the *Virbio spp* described, *V. anguillarum*, *V. alginolyticus*, *V. parahemolyticus* and *V. harveyi* have been described as pathogenic to penaeid shrimps (Vanderzant et al., 1970; Lightner and Lewis 1975; Lightner 1983, 1988; Takahashi et al., 1985 a). In addition a *Vibrio campbellii* - like bacterium was isolated from diseased hatchery reared larvae of *P. indicus* which was found to be highly pathogenic to larvae, post-larvae and adult animals (Hameed, 1989).

Cultured isolates caused mortalities of normal shrimp within 3 hours when added to experimental aquaria. In a subsequent study Lewis (1973) isolated *V. anguillareum* from moribund brown shrimp from an experimental pond in which mass mortalities had occurred. Isolates were pathogenic to normal adults within 24 hours following injection with foci of infection in the hepatopancreas.

Important aspects of virbiosis in penaeid shrimps summarised by Lightner (1983) and Lightner et al.,(1984) are as follows:

Infections may be chronic, subacute or acute and mortality may reach 100% in some cultured populations.

Most outbreaks are consequences of extreme stresses and opportunistic pathogens.

Isolates of *Vibrio spp* from shrimp may not always produce experimental infection, except when massive doses are injected.

Larval, post - larval, juvenile and adult shrimp may be infected.

Vibrio spp which infect shrimp are ubiquitous and have been reported from all major shrimp culture regions.

Vibrio sp and strains differ markedly in their virulence for penaeids, as they do for other hosts.

Luminescent Vibriosis:

Significant larval mortalities associated with luminescent vibriosis, caused by *V. harveyi* and *V. splendidus* were reported from *P. monodon* and *P. merguensis* hatcheries in Indonesia (Sunaryanto and Mariam. 1986), the Philippines (Baticados et al., 1991) and in Thailand, Tansutapanit and Ruangpan, (1987). Lavilla Pitogo et al.,(1990) reported that near shore sea water could be a major source of *V. harveyi* and *V. splendidus* for shrimp hatcheries in the Philippines Baticados et al.,(1991) tested 24 antibacterials against *V. harveyi* and *V. splendidus* in *P. monodon* larvae and showed that only chloramphenicol, sodium nifurstyrenate and the nitrofurans caused reasonable growth inhibition of the bacteria.

Miscellaneous vibrios and other bacteria:

Takahashi et al.,(1985 a) and Egusa et al.,(1988) reported serious *Vibrio sp.* epizootics in post- larval and juvenile *P. japonicus* in Japan. This condition, which existed since 1981, was characterized by cloudiness of the hepatopancreas in post-larvae and cloudiness of the muscle and brown spots in the gills and lymphoid organ in the juveniles (Takahashi

et al.,1985a).The serious epizootic in *P. japonicus* were controlled with oxytetracycline - medicated feed at 50 - 100 mg/kg body wt/day for 4 to 6 days(Takahashi et al., 1985b).In vitro vaccination of juvenile *P. japonicus* with Formalin killed *Vibrio sp* was found to provide some protection against subsequent challenge by live *Vibrio sp.* (Itami et al.,1989).

In Malaysia, Anderson et al.,(1988) reported heavy mortalities in market size (25 to 33 g) *P. monodon* associated with multifocal necrosis, hemocytic inflammation in the lymphoid organ, heart, gills, hepatopancreas, antennal gland, cuticular epidermis and in other connective tissues. Some haemocyte nodules contained Gram negative bacteria within the granulomas or within intracytoplasmic vacuoles. From the haemolymph of such shrimp, *V. alginolyticus*, *V. parahemolyticus* and *Pseudomonas sp.* were isolated.

Drugs/chemotherapy (antibiotics/antiseptic compounds):

Chemotherapeutics are drugs capable of affecting or killing bacteria. Antibiotics comes under this category.

According to Meyer (1991) 'Chemotherapy should be considered as an emergency or last - resort measure. Although chemicals may reduce the incidence of pathogens or control the abundance of facultative organisms, they also may have negative effects on desirable pond biota and on the flora of biological filters. Some chemicals may be hazardous to the user or leave undesirable or harmful residues in the culture animals'.

When preventive measures fail, it is necessary to treat diseases with antibiotics or other antibacterial chemicals. In many of the Asian countries, compounds like saponin, formalin, malachite green, treflan, chloramphenicol, oxytetracycline and furanace are being used. Such therapy is most practically applied in hatcheries, where high density of young ones are present in less volume of water.

Antibiotics:

Being chemically highly heterogeneous substances formed from fungi/bacteria or by synthesis, the antibiotics inhibit or kill other microbes without significant harm to the macro organisms.

The modes of action of antibiotics depend on the type of antibiotics. Some common modes are:

(i) Penicillins primarily act on the nucleotides. The synthesis of specific substances which form the membranes (*mucoproteins*) is thereby hindered; the synthesis and growth of bacterial cell membranes are also affected.

(ii) Polymyxin acts on the constituents of the protoplasmic membranes, so that their permeability is affected.

(iii) Chloramphenicol, streptomycin, erythromycin and the tetracyclines inhibit protein synthesis in the micro-organism.

(iv) Actinomycin affects the synthesis of RNA

Antimicrobial sensitivity and assay tests:

The selection of therapeutic agents for the treatment of any bacterial disease is obviously based on the susceptibility of the infecting strain and on the host tolerance to such drugs. In most microbiological laboratories bacterial susceptibility to different antibiotics is routinely determined by the

kirby - Bauer method (Bauer et al.,1966), more commonly known as the Disc Diffusion Test. In this method an antibiotic - impregnated paper disc placed on an agar medium releases the drug. creating a circular diffusion gradient since the antibiotic concentration decreases with distance from the disc, the size of the growth inhibition zone is used as a measure of the susceptibility of the bacterial strain tested.

The most important efforts in the improvement of sensitivity tests have been directed towards a standardised method that is generally acceptable. As a result, in respect of diffusion tests, Sweden has adopted the method of the International Collaborative Study (Ericsson and Sherris,1971);in the U.S.A. the Food and Drugs Administration has adopted the Kirby-Bauer technique (Bauer et al.,1966); in the UK the controlled disc method of Stokes and Ridgway (1987) is generally used.

In the classic diffusion technique a source of the antimicrobial agent is applied to the surface of the medium (Collins et al.,1989).The diffusion of the agent through the medium inhibits the growth of a sensitive organism growing in it or on it to a degree partially dictated by the susceptibility of the organism. It is well known, however,

that a number of other factors which influence the size of the zones of inhibition and these factors are to be controlled to attain consistent results. Plates poured flat with an even depth of medium throughout and all contain the same volume of medium, (for example, 15ml) for a 90mm petridish, gave consistent results. These techniques were standardised in the present investigations.

MATERIAL AND METHODS

I. Bacterial strain:

The bacteria used for the evaluation of influence of different inhibitory substances was initially isolated from moribund *Penaeus monodon* from culture ponds of Ramnad. The initial isolation was carried out from the hepatopancreas of the infected shrimp. Axenic cultures were maintained in Zobell marine agar slants and infectivity of the isolates was checked using healthy shrimps. In the laboratory, the pathogen was characterized as *Vibrio alginolyticus*.

II. Media:

Zobell marine agar:

Zobell marine agar supplied by Hi- Media with the following composition was used:

Ingredients	g/l
Peptone	5.0
Yeast extract	1.0
Ferric citrate	0.1
Sodium chloride	19.45
Magnesium chloride	8.8
Sodium sulphate	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 fluorate	0.0024
Ammonium nitrate	0.0016
Disodium phosphate	0.008
Agar	15.0

Final pH (at 25^{o} C) 7.6 + 0.2

Nutrient Agar:

Nutrient Agar used for present investigation was manufactured by Hi-Media. The following was the composition of the media:

Ingredients	g/l
Beef extract	3.0
Peptone	5.0
Sodium chloride	8.0
Agar	15.0
(pH at 25° C 7.3 + 0.2)	

Nutrient broth:

Nutrient broth supplied by Hi-Media was used with the following composition:

Ingredients	g/l
Beef extract	10.0
Peptone	10.0
Sodium chloride	05.0
(pH at 25° C 7.4 + 0.2)	

Table 1
Antibiotic discs and the tested concentrations

Antibiotics	Concentration
Amoxycillin	10 ug
Augmentin	10 mcg
Bacitracin	10 mcg
Cephalexin	30 mcg
Chloramphenicol	30 ug
Ciprofloxacin	30 mcg
Clindamycin	2 mcg
Cloxacillin	1 mcg
Co-Trimaxazole	25 mcg
Erythromycin	15 mcg
Gentamycin	10 ug
Kanamycin	30 ug
Linomycin	2 mcg
Methicillin	5 mcg
Neomycin	30 ug
Norfloxacin	10 mcg
Oleandomycin	15 mcg
Oxytetracycline	30 mcg
Penicillin	10 units
Streptomycin	10 ug
Tiamulin	30 ug
Tobramycin	10 mcg

Table 2
Antiseptic chemicals used in the Zone Inhibition Assay

Antiseptic chemicals	Tested concentrations
Copper sulphate	0.1, 0.5, 1.0 %
Potassium permanganete	0.1, 0.5, 1.0, 5.0 %
Potassium di chromate	0.1, 0.5, 1.0, 5.0, 10 %
Formalin	10, 20, 50 %
Malachite green	100, 200, 500, 750, 1000 ppm
Methylene blue	1, 10, 50, 100, 200, 500 ppm
Acriflavin	1, 10, 50, 100, 200, 500 ppm
Acid fuschin	100, 200, 500 ppm
Light green	100, 200, 500 ppm
Virkon	0.5, 1, 2, 5%

In 250ml conical flasks 100ml of Nutrient broth was taken and autoclaved. A loopful of bacterial culture from a slant was used to inoculate the broth after sufficient cooling under aseptic conditions. It was then incubated for 24 h at desired temperatures for broth culture experiments.

III. Experiments on Antibiotic sensitivity:

Antibiotic sensitivity of the *V. alginolyticus* was examined by using antibiotic sensitivity discs (Hi-Media). Details of the antibiotic discs used and their concentrations are given in Table 1. Nutrient agar plates were prepared and the inocula (0.5ml) was spread on the surface. By using sterilized forceps, antibiotic discs were carefully placed over it and incubated at 37 C. Diameter of the inhibition zone was measured at 6 h intervals using a divider. Readings were taken upto 24 h and plotted on the arithmetic graph.

IV. Experiments on Zone Inhibition Assay using antibacterial chemicals:

The antibacterial chemicals used and their concentrations are given in Table 2.

Nutrient agar after autoclaving was poured in petri plates. In a 90mm petri plate, about 20ml of medium was poured and kept undisturbed for solidification. After solidification of media, 0.5ml of broth culture was spread over the media using a L- shaped rod. Wells of 15mm dia. were bored. In each petri plate 4 wells were bored. The bottom of the wells were sealed using soft agar. Each chemical at different concentrations was added drop by drop in the respective well and were immediately incubated at 37^{o} C.

Area of the inhibition zones formed was calculated based on the radius measurements carried out at every six hours intervals. The results were expressed in arithmetic graphs.

V. Experiments on inhibition of growth of *Vibrio alginolyticus* in broth culture:

An EC - made spectrophotometer was used to ascertain the inhibition of growth of *V. alginolyticus* by a disinfectant Virkon. The composition of Virkon (manufactured by Rakshak pharmacuetical) is given below:

Composition of Virkon	Concentration
Sodium chloride salt containing Potassium monophosphate Potassium Hydrogen Sulphate/ Potassium Sulphate	49.8%w/w
Buffer and Excipients Colour	q.s to 100%

Seven, 500ml conical flasks containing 100ml Nutrient broth were used. Of these, one was used as control. Six different concentrations of Virkon viz., 0.01, 0.1,0.5,1.0, 2 and 5% were studied.

After inoculating all the conical flasks with the same amount of bacterial suspension (2.5ml), the OD was measured at 660 nm. Then the flasks were inoculated at 25 C for 6 h and again OD was measured. Immediately, constant amount of Virkon at different concentrations were added except for the control. After 6 h OD was taken and this was continued upto 36 h at 6 h intervals.

VI. Experiments on extraction and testing of natural products from marine organisms:

Sponge extract:

Sponge, *Spongia officinalis* was collected from the Gulf of Mannar. The extract was prepared by methanol - chloroform method. Samples obtained were used for zone inhibition assay and the experiments were conducted at 25 C, room temperature and 37 C.

Seaweed extract:

Seaweeds, *Gracilaria sp.* and *Gelidiella sp.* were collected from the Gulf of Mannar and dried under shade. The dried seaweeds were powdered using a mixer. The sieved sample was kept in the thimble and placed inside the sample holder of the Soxlet unit. Ether was poured into the flask for heating. The apparatus was set and cold water was circulated through the condenser. The flask was heated at 60 C using a heating mantle. Ether evaporated and escaped into the sample chamber and then to the condenser. The process was repeated for four times and then the heating flask was removed. The resultant sample was used for zone inhibition studies.

Experiments on Neem oil:

Neem oil prepared from the kernal, obtained as a gift from Dr. J. Muthukrishnan, Madurai Kamaraj University was used to study the growth inhibition of *V. alginolyticus*. Different concentrations viz., 0.01, 0.1, 0.5, 1, 2 & 5 ppm were tested. Zobell marine medium was used for the zone assay studies. Measurement of growth inhibition was studied using a spectrophotometer.

RESULTS

The results of influence of antibiotic, antiseptic and natural (bioactive) substances on the growth inhibition of shrimp pathogenic *Vibrio alginolyticus* are presented in this section.

Influence of different antibiotics on the inhibition of Vibrio alginolyticus:

The results of antibiotic sensitivity using sensitivity discs of different concentrations are given in Table 3. It was found that *Vibrio alginolyticus* was sensitive to chloramphenicol at 30 ug level. Co-Trimaxazole at the concentration of 25 mcg ranked second in inhibiting growth of the bacteria. The other antibiotics such as Ciprofloxacin, Erythromycin, Streptomycin, Norfloxacin, Clindamycin, Amoxyllin, Kanamycin, Augmentin, Cloxacillin, Gentamycin and Neomycin were found to moderately inhibit *V. alginolyticus* at their respective concentrations (Table 3). However, *V. alginolyticus* was found to be resistant to Bacitracin, Cephalexin, Linomycin, Methicillin, Oleandomycin, Oxytetracycline, Penicillin, Tiamulin and Tobramycin. In almost all the antibiotics which exhibited

Table 3

Influence of different antibiotics on inhibition of growth of *V. alginolyticus*

Antibiotics	Conc.	Dia. (mm) of zone at different hours				
		0	6	12	18	24
Amoxycillin	10 ug	-	12	12	16	16
Augmentin	10 mcg	-	11	11	11	11
Bacitracin	10 mcg	-	-	-	-	-
Cephalexin	30 mcg	-	-	-	-	-
Chloramphenicol	30 ug	-	22	22	22	22
Ciprofloxacin	30 mcg	-	15	15	15	15
Clindamycin	2 mcg	-	13	13	13	13
Cloxacillin	1 mcg	-	10	10	10	10
Co-Trimaxazole	25 mcg	-	20	20	20	20
Erythromycin	15 mcg	-	14	14	14	14
Gentamycin	10 ug	-	10	10	10	10
Kanamycin	30 ug	-	12	18	18	18
Linomycin	2 mcg	-	-	-	-	-
Methicillin	5 mcg	-	-	-	-	-
Neomycin	30 ug	-	10	10	10	10
Norfloxacin	10 mcg	-	13	13	13	13
Oleandomycin	15 mcg	-	-	-	-	-
Oxytetracycline	30 mcg	-	-	-	-	-
Penicillin	10 units	-	-	-	-	-
Streptomycin	10 ug	-	14	14	14	14
Tiamulin	30 ug	-	-	-	-	-
Tobramycin	10 mcg	-	-	-	-	-

inhibition on growth of *Virbio*, detectable zone was noticed from 6 h of incubation at 37 C. The zone area increased in a few antibiotics after 12 h while in some, the zone area did not increase after the observed area at 6 h of incubation. The pattern of zone inhibition at different time intervals by the tested antibiotics is represented in Fig.1.

Experiments on inhibition of Vibrio alginolyticus using antiseptic compounds:

Vibrio alginolyticus responded differently with the different antiseptic compounds tested during the course of the present investigation. The results of concentrations of antiseptic compound influencing inhibition of *Vibrio alginolyticus* are presented in Tables 4 to 11.

Copper sulphate at 0.5% level was found to initiate inhibiting the bacteria. Detectable zone of inhibition was noticed only after 6 h of incubation at 37 C. At 0.1% level there was no inhibition even after incubation for more than 24 h (Table 4 & Fig.2).

Potassium permanganate, Acid fuschin and Light green did not inhibit the growth even at higher concentrations tested (Vide Table 2).

Fig.1.
Pattern of inhibition of *V. alginolyticus* by different antibiotics.

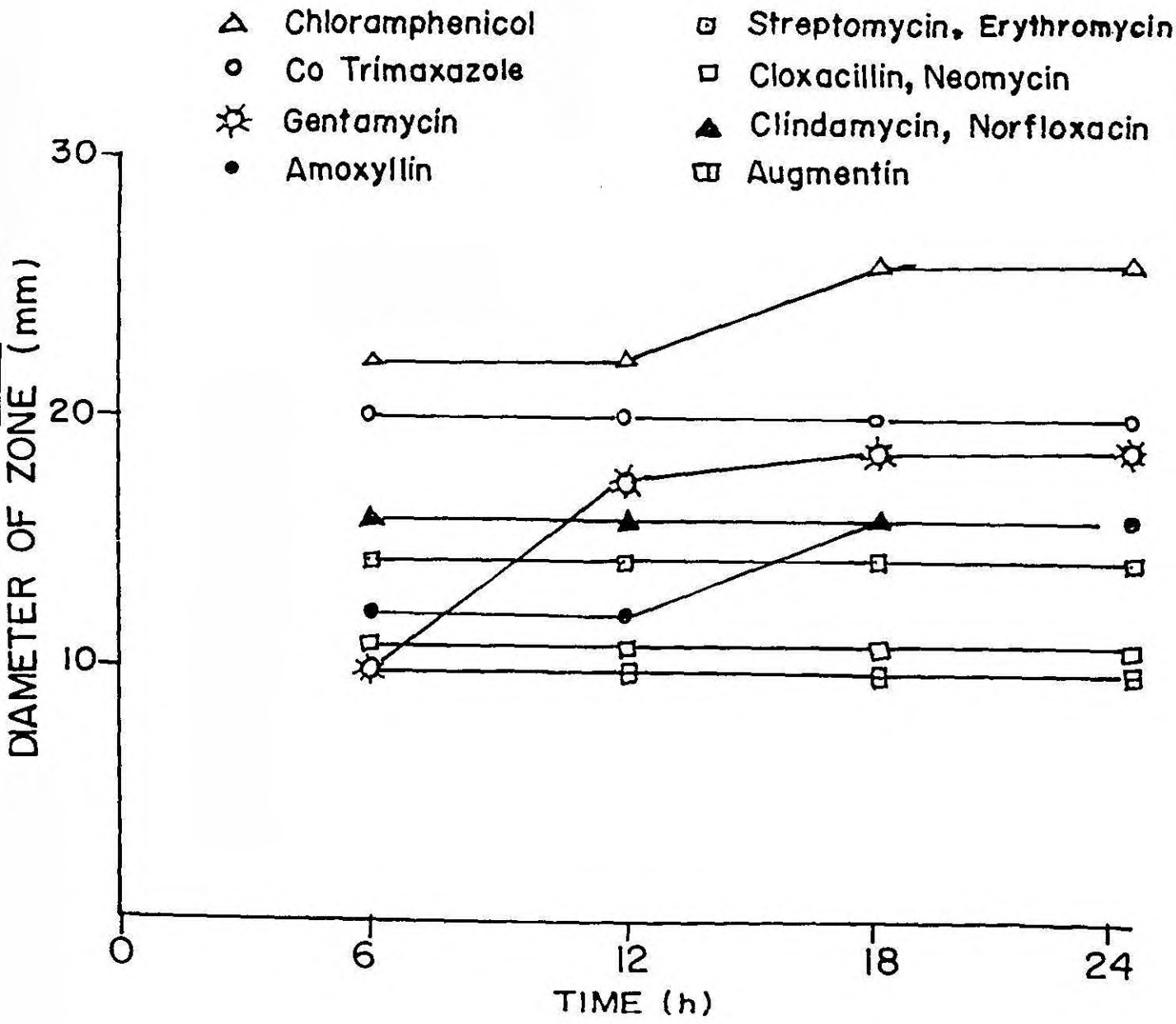

Chloramphenicol
Co Trimaxazole
Gentamycin
Amoxyllin
Streptomycin, Erythromycin
Cloxacillin, Neomycin
Clindamycin, Norfloxacin
Augmentin
DIAMETER OF ZONE (mm)
30
20
10
0
6
12
18
24
TIME (h)

Plate 1. Petridish showing inhibition zones by Octodisc.

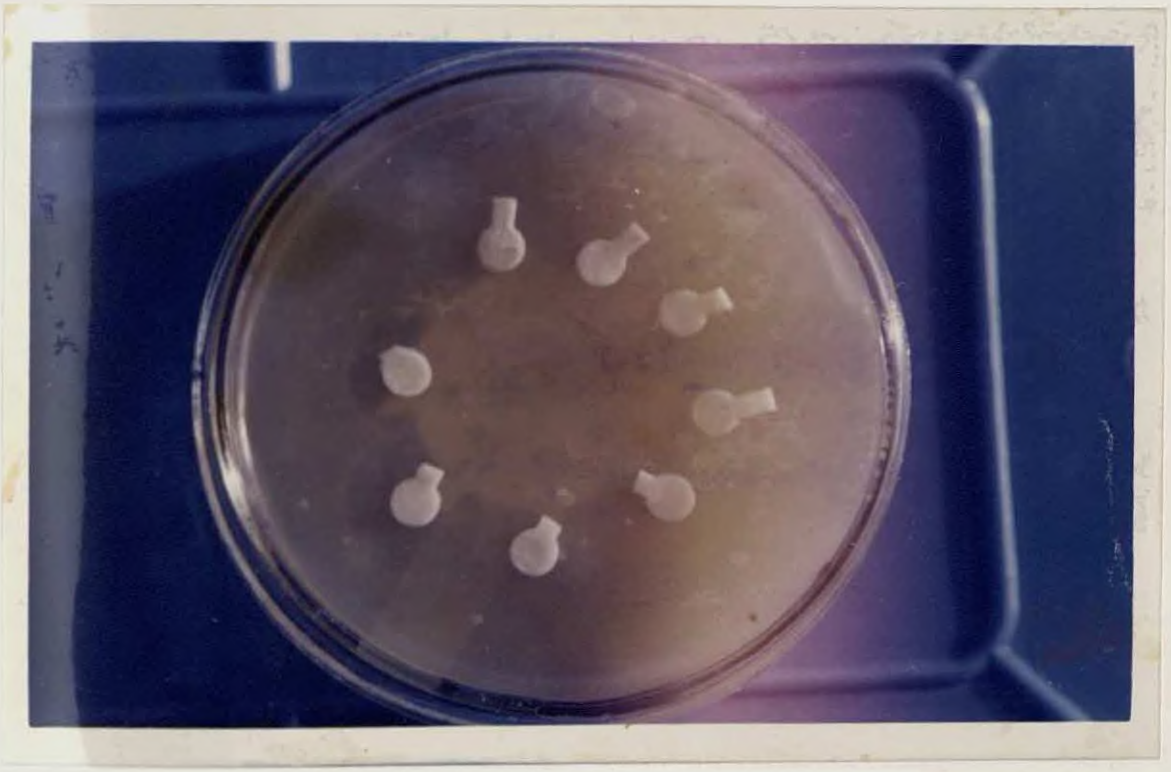


Fig.2.
Inhibition of V. alginolyticus by Copper sulphate.

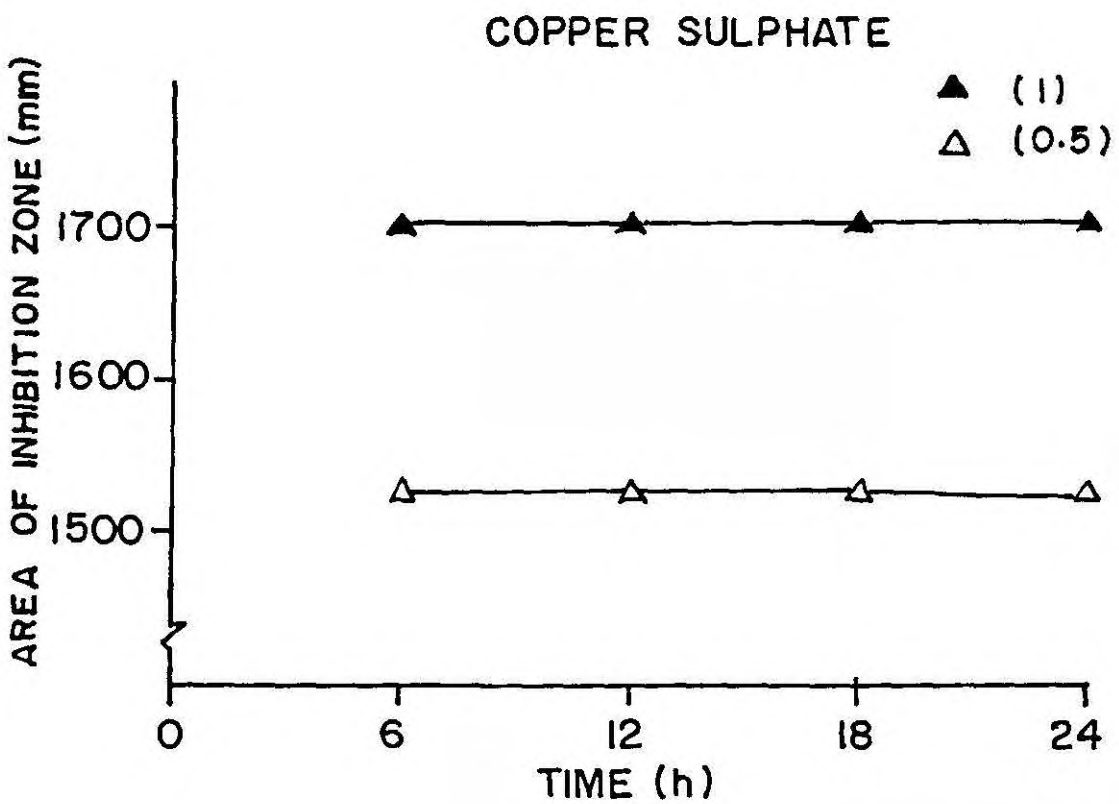

COPPER SULPHATE
(1)
(0.5)
AREA OF INHIBITION ZONE (mm)
1700
1600
1500
0
6
12
18
24
TIME (h)

Table 4

Effect of different concentrations of Copper sulphate on *V. alginolyticus*

Conc.	Zone area (sq. mm) at different hours				
(%)	0	6	12	18	24
0.1	-	-	-	-	-
0.5	-	-	1557	1557	1557
1.0	-	-	1708	1708	1708

Potassium di chromate started inhibiting the growth before 6 h of incubation. The zone diameter increase was found to be proportional to the concentration of the chemical. At 0.1% and 0.5%, there was no inhibition while at 1% level onwards inhibition was noticed. It was interesting to note that there was no increase in the zone diameter from 6 h of incubation to 24 h of incubation as could be seen from Table 5 and Fig.3.

Formalin at tested concentrations was found to completely inhibit the bacterial growth (Table 6).

The antiseptic compound Malachite green which is widely used in hatcheries was found to inhibit the growth of *Vibrio alginolyticus* from 500 ppm level onwards. The inhibition zone was directly proportional to the tested concentrations viz., 500, 750, 1000 ppm. The results are given in Table 7. Fig.4 gives the pattern of inhibition by malachite green.

Acriflavin at 50 ppm level onwards inhibited the growth of *Vibrio alginolyticus*. Acriflavin used as an antiseptic compound in fish and shellfish bearing waters. The pattern

Table 5

Effect of different concentrations of Potassium-di-chromate on growth of *V. alginolyticus*

Conc.	Inhibition zone area (sq.mm) at different hrs				
(%)	0	6	12	18	24
0.1	-	-	-	-	-
0.5	-	-	-	-	-
1.0	-	314	314	314	314
5.0	-	577	577	577	577
10	-	785	785	785	785

Fig.3.
Zone of inhibition of *V. alginolyticus* by Potassium-dichromate.

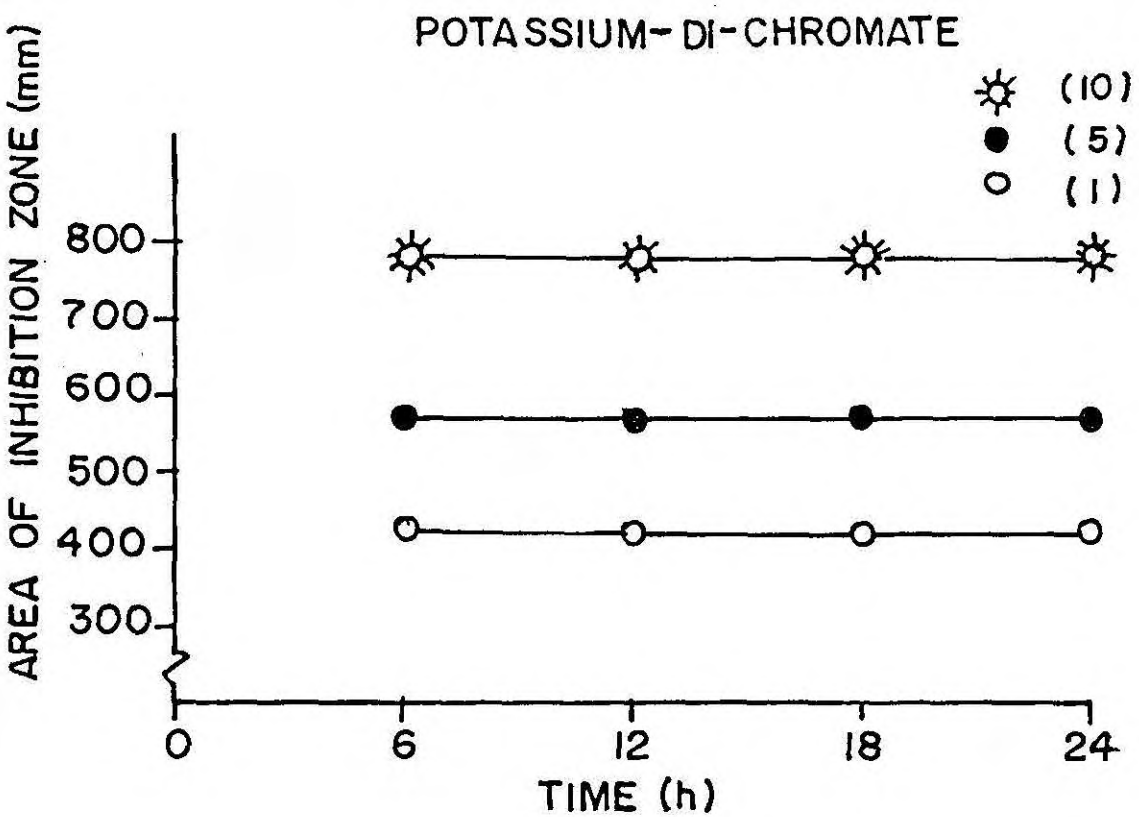

POTASSIUM-DI-CHROMATE
(10)
(5)
(1)
AREA OF INHIBITION ZONE (mm)
800
700
600
500
400
300
0
6
12
18
24
TIME (h)

Plate 2. Petridish showing inhibition by Potassium-dichromate

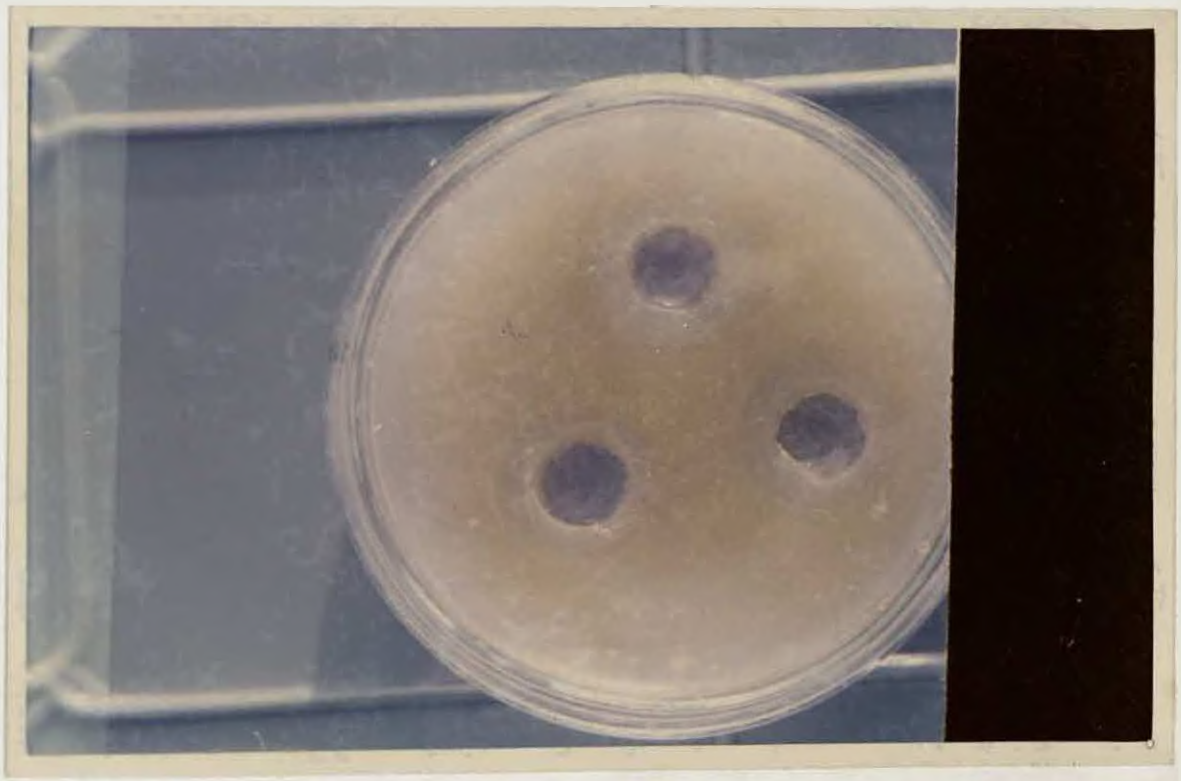


Table 6

Effect of different concentrations of Formalin on inhibition of *V. alginolyticus*

conc.	Relative zone inhibition at different hours				
(%)	0	6	12	18	24
10	+++	+++	+++	+++	+++
20	+++	+++	+++	+++	+++
50	+++	+++	+++	+++	+++

+++ Highly sensitive

Table 7

Effect of different concentrations of Malachite green on inhibition of *V. alginolyticus*

Conc.	Zone inhibition area (sq.mm) at different hrs				
(ppm)	0	6	12	18	24
100	-	-	-	-	-
200	-	-	-	-	-
500	-	169	169	169	169
750	-	238	238	238	238
1000	-	314	314	314	314

Fig.4.
Influence of Malachite green on the inhibition of growth of *V. alginolyticus*.

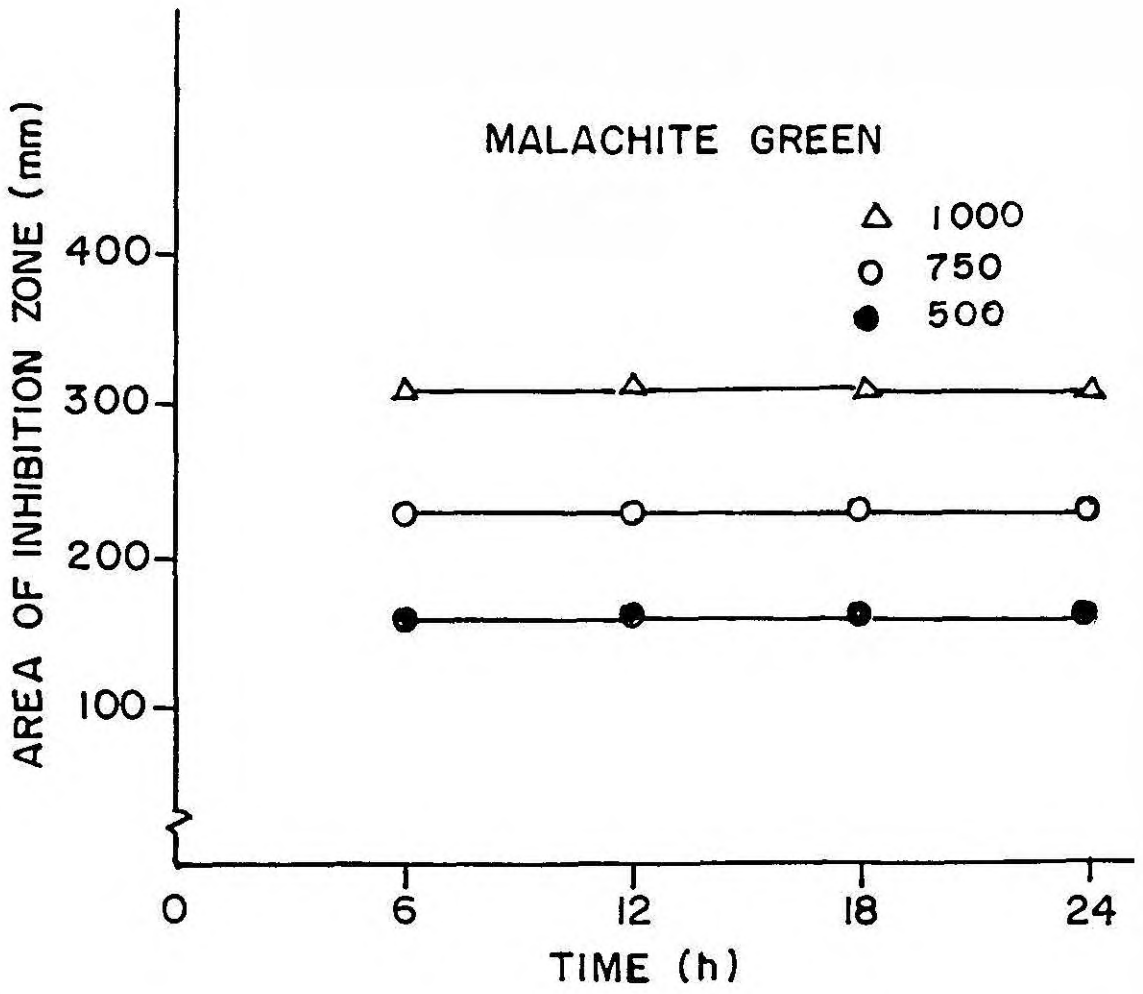

MALACHITE GREEN
1000
750
500
AREA OF INHIBITION ZONE (mm)
400
300
200
100
0
6
12
18
24
TIME (h)

Plate 3. Petridish showing inhibition zones by Malachite green.

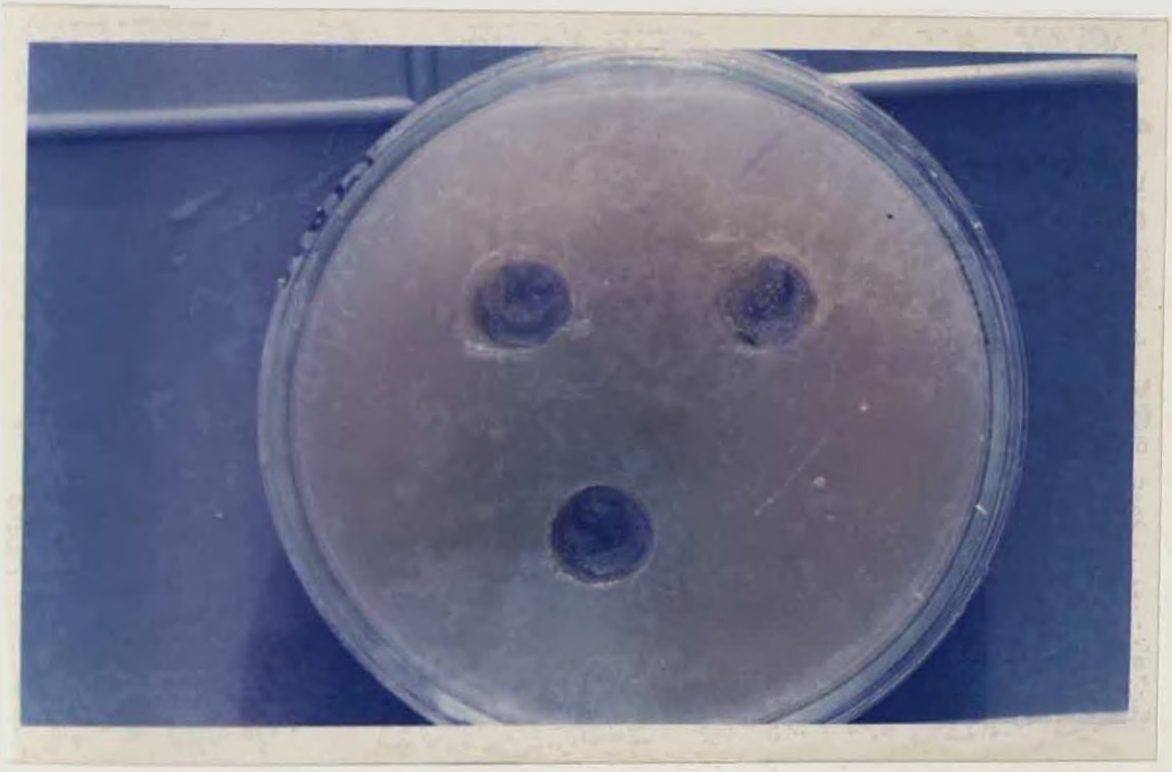


Table 8

Effect of Acriflavin concentrations on inhibition of *V. alginolyticus*

Conc.	Inhibition zone area (sq.mm) at different hrs				
(ppm)	0	6	12	18	24
1.0	-	-	-	-	-
10	-	-	-	-	-
50	-	138	138	138	138
100	-	314	314	314	314
200	-	785	785	785	785
500	-	785	785	785	785

of zone inhibition is presented in Fig.5.

In the case of methylene blue, the inhibition of *V. alginolyticus* was observed from 10 ppm conc. onwards (Table 9). The area of inhibition zone was found to be proportional to the concentration of the chemical as could be noted from Table 9 and Fig.6. The zone area did not increase after the initial 6 h period to 24 h of incubation.

The antiseptic compound Virkon was found to inhibit at the tested low concentration of 0.5% level. Detectable zone was noticed after 6 h of incubation at 37 C. The results are given in Table 10. As could be seen from Fig.7, the area of zone was proportional to the concentration at different hours of incubation except at higher concentration of 5% tested there was an initial lag period upto 24 h and then a spurt in the activity.

Fig.5.
Pattern of V. alginolyticus zone inhibition by Acriflavin.

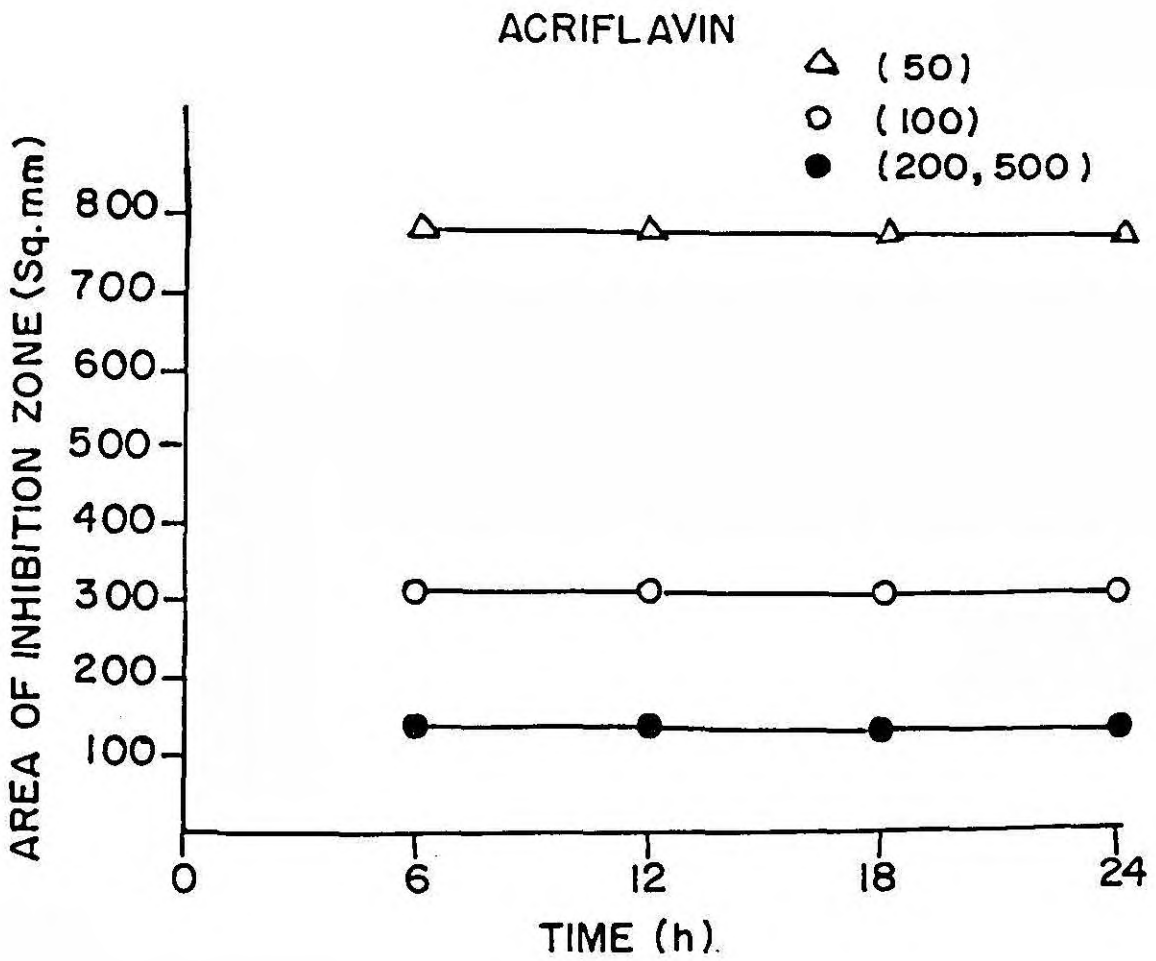

ACRIFLAVIN
(50)
(100)
(200, 500)
AREA OF INHIBITION ZONE (Sq.mm)
800
700
600
500
400
300
200
100
0
6
12
18
24
TIME (h)

Plate 4. Petridish showing inhibition zones by Acriflavin

Table 9

Effect of Methylene blue concentrations on growth inhibition of *V. alginolyticus*

Conc.	Inhibition zone area (sq.mm) at different hrs				
(ppm)	0	6	12	18	24
1.0	-	-	-	-	-
10	-	106	106	106	106
50	-	169	169	169	169
100	-	314	314	314	314
200	-	395	395	395	395
500	-	395	395	395	395

Fig.6.
Pattern of V. alginolyticus zone inhibition by Methylene blue.

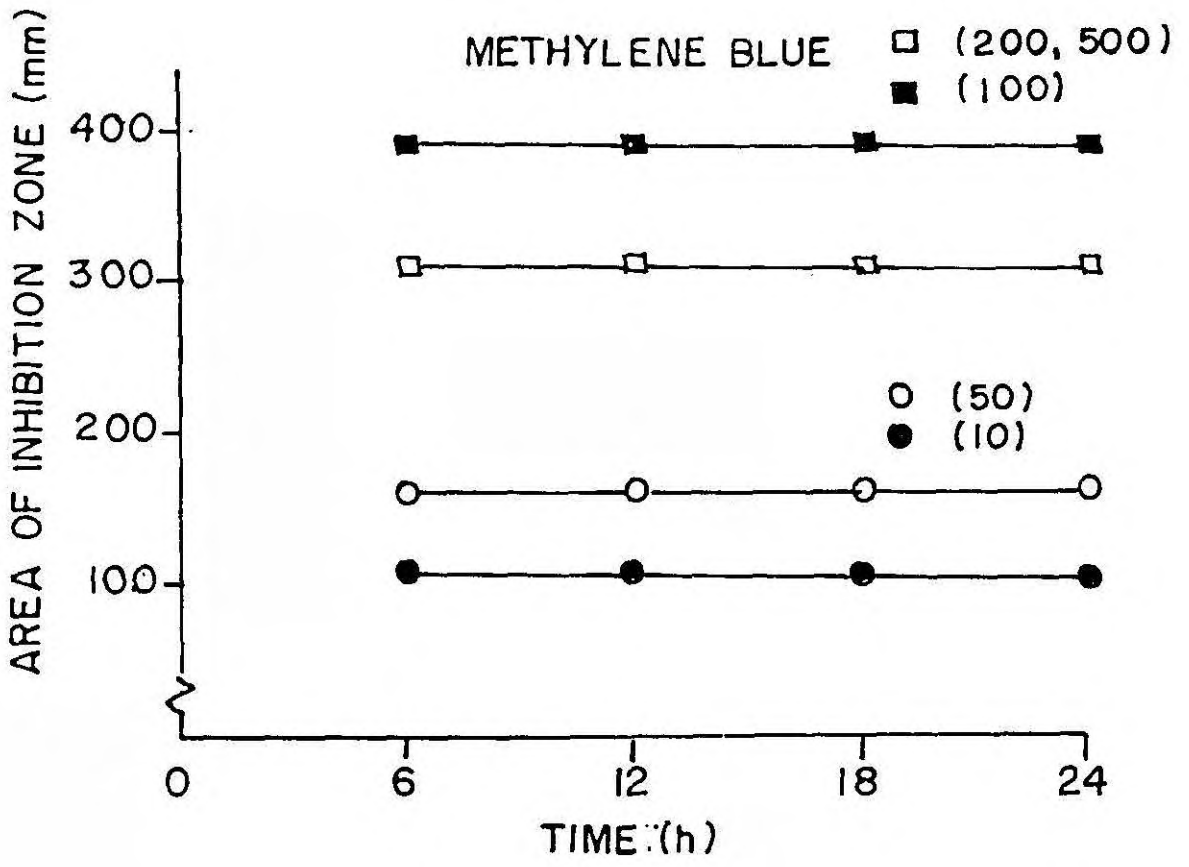

METHYLENE BLUE
(200, 500)
(100)
(50)
(10)
AREA OF INHIBITION ZONE (mm)
400
300
200
100
0
6
12
18
24
TIME (h)

Plate 5. Petridish showing inhibition zones by Methylene blue

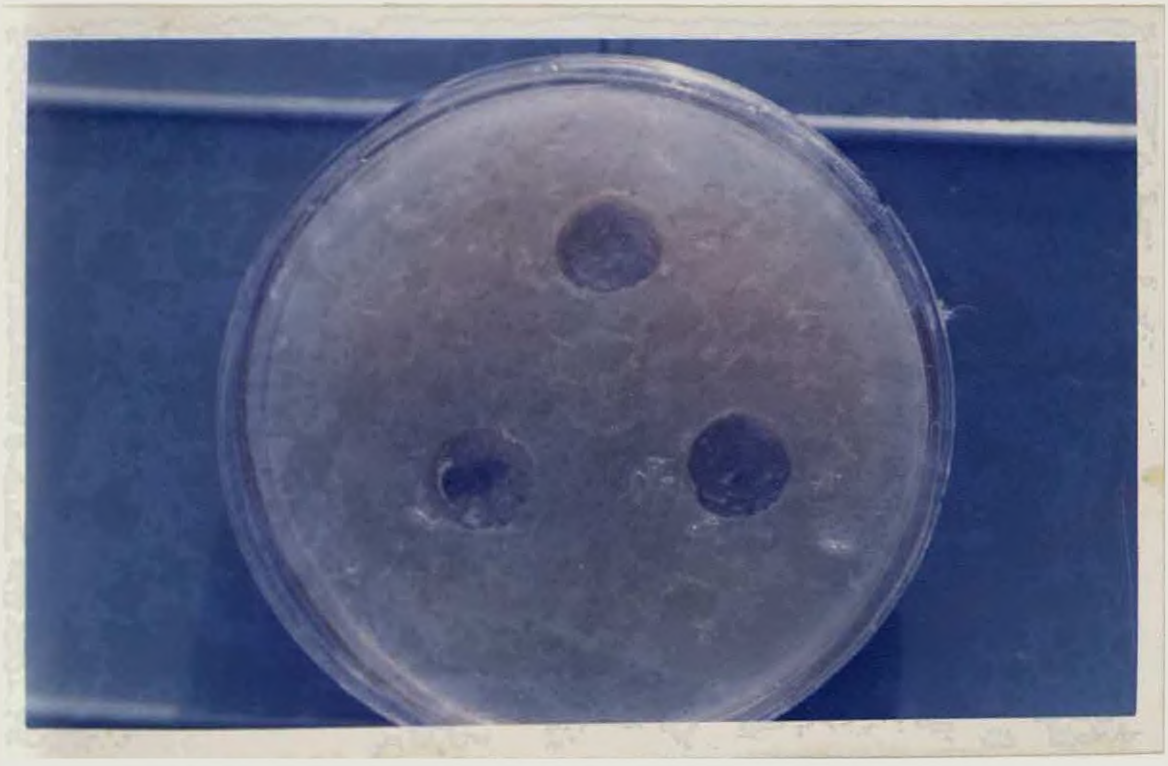


Fig.7.
Inhibition of *V. alginolyticus* by Virkon.

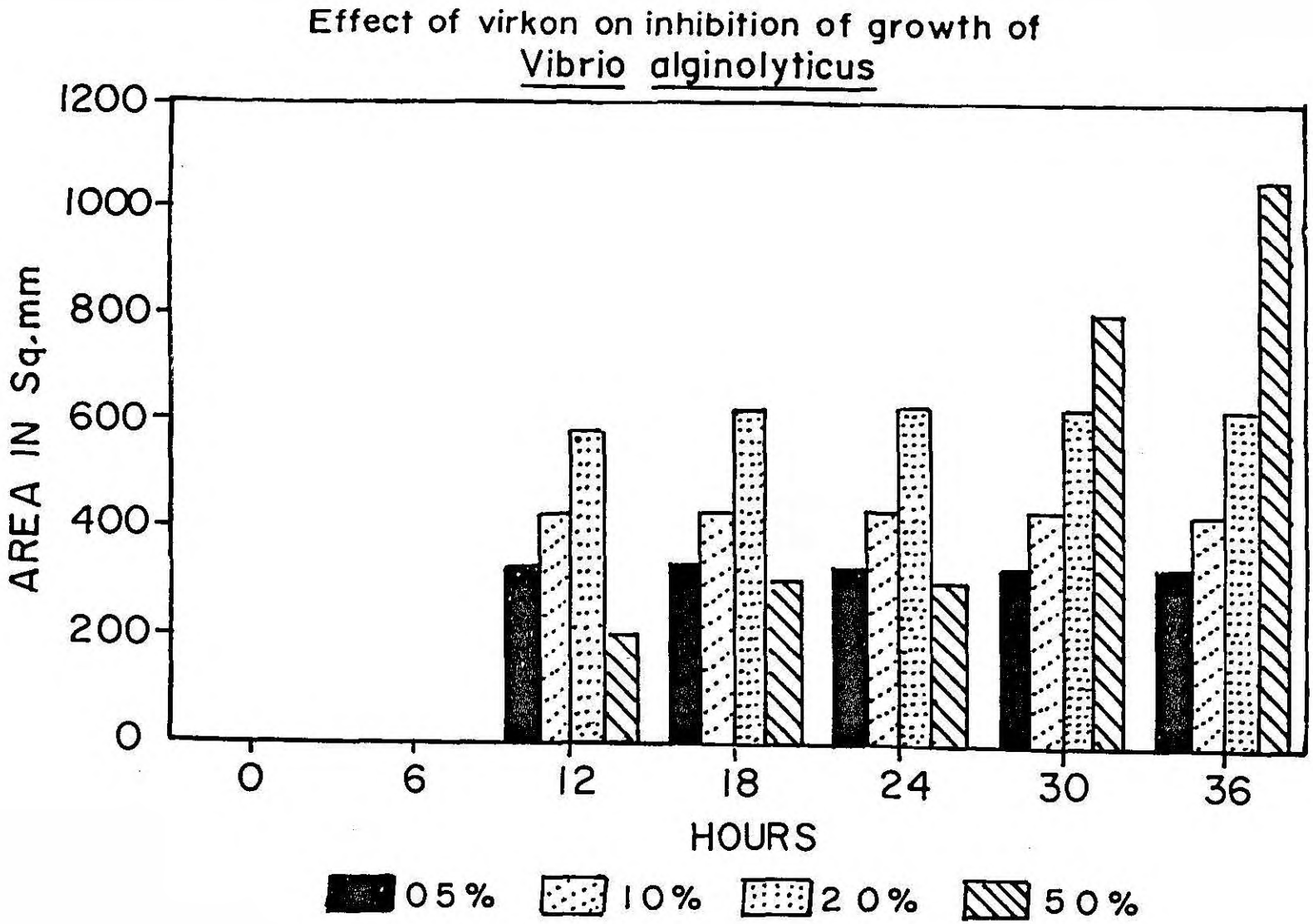

Effect of virkon on inhibition of growth of
Vibrio alginolyticus
AREA IN Sq-mm
1200
1000
800
600
400
200
0
0
6
12
18
24
30
36
HOURS
05%
10%
20%
50%

Fig.8.
Growth inhibition of *V. alginolyticus* by Virkon.

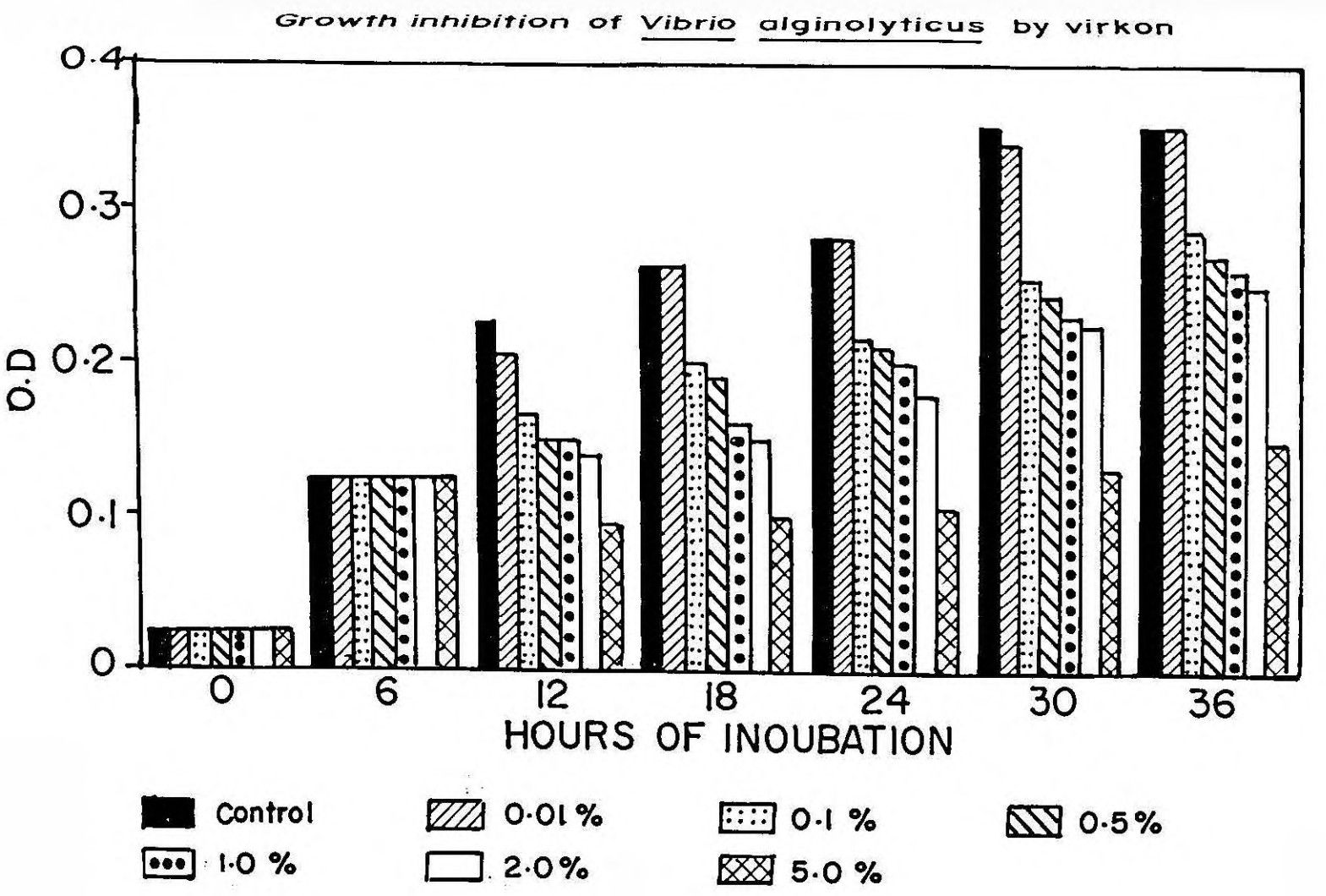

Growth inhibition of Vibrio alginolyticus by virkon
0·4
0·3
0·2
0·1
0
O.D
0
6
12
18
24
30
36
HOURS OF INOUBATION
Control
0·01 %
0·1 %
0·5%
1·0 %
2·0%
5·0 %

Table 10

Inhibition of *Vibrio alginolyticus* by Virkon (Inhibition zone assay)

% of VIRKON	Inhibition Zone area (sq.mm) at different hours						
	0	6	12	18	24	30	36
0.5	-	-	325	330	330	330	330
1	-	-	422	430	430	430	430
2	-	-	575	625	625	625	625
5	-	-	200	300	300	800	1050

Table 11

Inhibition of growth of *V. alginolyticus* by Virkon (spectrophotometric method)

% of VIRKON	O.D at different hours						
	0	6	12	18	24	30	36
C	0.025	0.125	0.225	0.260	0.280	0.355	0.355
0.01	0.025	0.125	0.205	0.260	0.280	0.344	0.355
0.1	0.025	0.125	0.165	0.200	0.215	0.255	0.285
0.5	0.025	0.125	0.150	0.190	0.210	0.245	0.270
1	0.025	0.125	0.150	0.160	0.200	0.230	0.260
2	0.025	0.125	0.140	0.150	0.180	0.225	0.250
5	0.025	0.125	0.095	0.095	0.105	0.130	0.150

Experiments on inhibition of growth of *V. alginolyticus* in broth cultures:

The antiseptic compound Virkon was found to inhibit the growth of *V. alginolyticus*. The O.D values at different hours indicated that right from 0.1% level onwards bacterial density was reduced (Table 11). The growth inhibition was directly proportional to the tested concentrations as could be seen from Fig.8.

Experiments on growth inhibition of *Vibrio* by natural (bioactive) products:

The natural products tested were found to behave differently on the growth inhibition of *V. alginolyticus*.

Sponge extract:

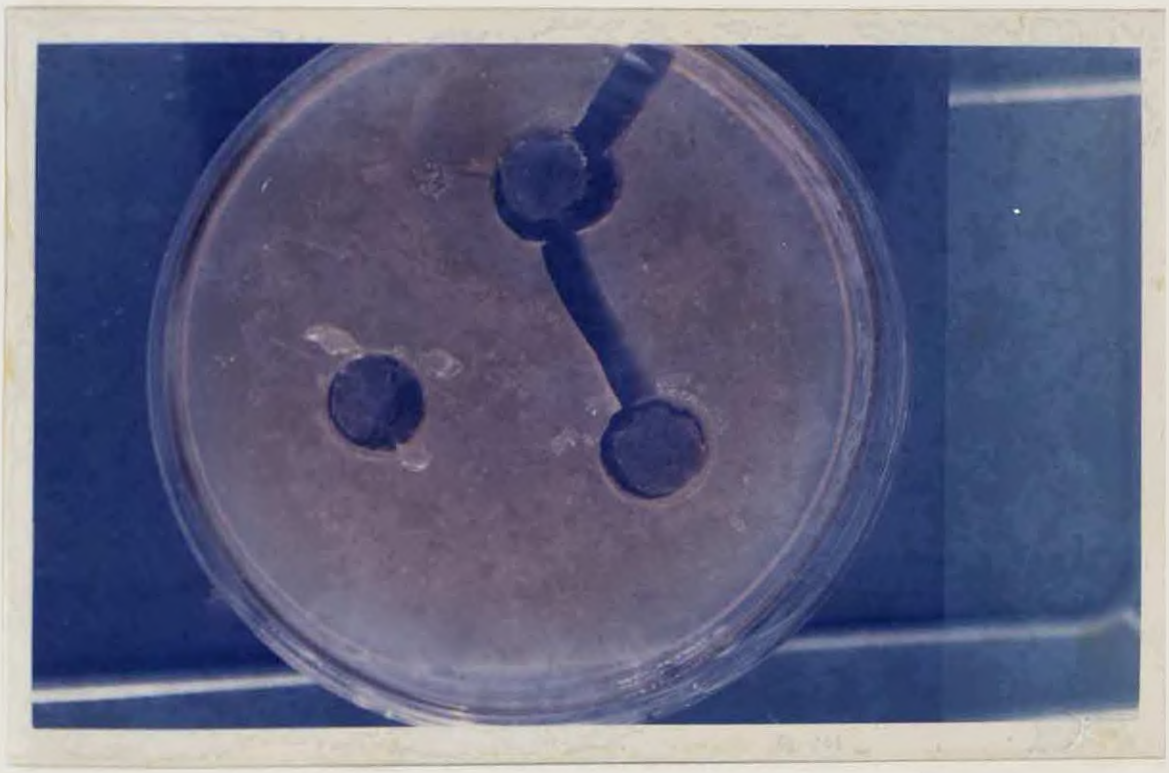
The results of inhibition of growth of *V. alginolyticus* by sponge extract is given in Table 12. It is interesting to note that the sponge extract was active only at lower temperature of 25 C and not at room temperature as well as at 37^{o} C.

Table 12

Inhibition of growth of *V. alginolyticus* by sponge extract at different temperatures

Duration	Inhibition zone area (sq.mm) at different temperatures		
	25 C	RT	37 C
6	-	-	-
12	-	-	-
18	1413	-	-
24	1413	-	-

Plate 6. Petridish showing inhibition zones by sponge extract



Seaweed extract:

The seaweed extract did not show any inhibition on *V. alginolyticus*.

Neem Extract:

In the zone assay no positive results were obtained. Results of O.D observations are given in Table 13. It could be noted that there was no retardation of growth of *V. alginolyticus* by neem extract.

Table 13

Optical density readings at 660 nm of culture of *V. alginolyticus* added with different concentrations of Neem oil

Conc.	O.D at different time intervals		
	0	6	12
control	0.020	0.080	0.125
0.01	0.025	0.075	0.125
0.1	0.010	0.060	0.110
0.5	0.025	0.070	0.120
1	0.025	0.070	0.125
2	0.025	0.070	0.120
5	0.025	0.085	0.115

DISCUSSION:

In the antibiotic sensitivity tests *vibrio sp.* was found to be highly sensitive to chloramphenicol. According to Baticados et al.,(1990)luminous bacteria (*V. harveyi* and *V. splendidus*) showed varied responses to chloramphenicol. The minimum inhibitory concentrations of chloramphenicol against *Vibrio* isolated from diseased larvae have been determined as 5 mg/l (Hameed and Rao, 1994). However, in the present investigations, very low concentration (30 ug) was found to inhibit shrimp pathogenic *V. alginolyticus*. Hence, this antibiotic compound can be recommended for hatchery purposes. While using chloramphenicol certain precautions are to be taken. Earlier reports of experiments indicated that chloramphenicol when added to the culture systems adversely affected the *Chaetoceros* population. In Thailand a comparison of resistant bacteria isolated between 1988 - 1991 and 1992, indicated that *Vibrio* bacteria were increasing their resistance to chloramphenicol. Resistance to chloramphenicol was also reported earlier but by least percentage (15.8%) of strains (Rosily and Sreekumari, 1987). Although different strains were not studied in the present investigations, the *V. alginolyticus* was found to be highly susceptible to chloramphenicol.

The bacteria (*Vibrio*) was highly resistant to Oxytetracycline. The low sensitivity of some luminous bacteria (*V. harveyi* and *V. splendidus*) to Oxytetracycline were earlier reported by Baticados et al.,(1990).In the present study, Oxytetracycline used was of 30 ug concentration. MICs of Oxytetracycline were found out as 0.39 and 100 ppm. This confirmed the report of Ruangpan and Kitao (1992), who found that the MIC value of OTC against 205 isolated *Vibrio* sp. from diseased shrimp in Thailand ranged between 0.2 and 100 ppm. The minimum inhibitory concentrations of OTC against *Vibrio* isolated from diseased larvae have been determined as 150 mg/l (Hameed and Rao , 1994). Some of the earlier reports, one from Thailand (1988-1991, and 1992), and another from India (Rosily et al.,1987) indicated the resistance showed by a few *Vibrio* strains. The present investigation therefore, confirms the general earlier observation that *Vibrio sp.* are less sensitive to oxytetracycline. Based on the results, *V. alginolyticus* could be taken as another bacterial species resistant to Oxytetracycline.

The antibiotic Tiamulin (30 ug), tested in the present investigation was less effective. The effect of Tiamulin on *Vibrio anguillarum* studied indicated that the bacteria was sensitive at an MIC range of 1.6 to 6.25 ppm. Although the MIC was not studied in the present investigations, the trend of inhibition when compared with other antibiotics is negative.

Oleandomycin used was not at all effective, but Chan and Lawrence (1974) reported that the combination of OTC-Oleandomycin in reducing bacterial populations in larval shrimp cultures was effective.

Eventhough many antibiotics are effective in controlling Vibriosis and other bacterial diseases, the chance of multiple drug resistance, generation of drug resistant bacterial strains, antibiotic residues in the cultured animals are to be studied in detail.

The antibiotics principally act via the genetic apparatus of the microbes on their division, thus mainly as bacteriostatic agents. The bacteriostasis is reversible; the multiplication can be carried on afresh, if the action of the antibiotics ceases. However, a lytic effect is

possible, or the enzymatic and oxidation processes may be affected, so that the result is a bactericidal action. This action is irreversible and is a function of the dosage (Schaperclaus, 1991).

In actual practice, it is important that the practically attainable therapeutic level of antibiotics leads only to bacteriostasis. Additionally, under this condition, all normal defense functions of the macro organism should be intact. No effects can be obtained even with the best antibiotics, if the host organism is so weakened due to infections or other effects that the normal defense functions are restrained.

Antiseptics:

Among the antiseptic chemicals, Copper sulphate was very effective even at 0.5 % concentration. Aqatrine (manufactured by applied biochemists, IMC.), a chelated Copper sulphate compound is registered and approved by US Environmental Protection Agency (EPA) for use in fish and shrimp culture (Bell, 1992).

Potassium di chromate was effective at 1.0 % level. But the degree of inhibition was not much higher when comparing to Copper sulphate. In the case of Formalin total inhibition was recorded at all the tested concentrations. Formalin has also been approved by the FDA or EPA (Bell, 1992).

The activity of Malachite green ,Acriflavin, Methylene blue towards inhibition of *V. alginolyticus* was in the following order:

Methylene blue > Acriflavin > Malachite green

Malachite green, Acriflavin, Methylene blue are generally used in fish and shellfish hatcheries as well as culture system. The present findings indicate that these chemicals are also active against controlling shrimp pathogenic *V. alginolyticus* so that they can be recommended. Potassium permanganate was not effective even at higher concentration (10%). Similar is the case with Acid fuschin and Light green.

The disinfectant Virkon was showing increased activity with increasing concentrations. There is no comparative data available on inhibition by Virkon against other shrimp

pathogenic species of Vibrio.

Natural products:

The seaweed extract, were known to show inhibitory action against Gram negative bacteria (Rao, 1982). Padmini et al. (1990) reported inhibitory effect of extracts of red algae against many bacterial species. The growth of Vibrio was also found to be inhibited by Hypnea musciformis (Avelyn, et al. 1991). The natural compounds have several advantages over the antimicrobial chemical substances in combating bacterial diseases in aquaculture. The experimental results indicated the possibilities of utilisation of natural (bioactive) substances of terrestrial/marine origin.

SUMMARY

In the present investigations, influence of several antibiotic, antiseptic and natural substances on the inhibition of shrimp pathogenic V. alginolyticus was studied.

Out of the 22 antibiotics tested, Vibrio alginolyticus was found to be sensitive to 10 and resistant to the rest.

Among the sensitive antibiotics , Chloramphenicol, Co-Trimaxazine (3.0 mcg) were found highly effective.

Antibiotics such as Augmetin (10 mcg) Amxycillin (10 ug) Streptomycin(10 mcg) ,Gentamycin (10 ug), Kanamycin (30 ug),Erythromycin (15 ug), Norfloxacin (10 mcg), Clindamycin(2 mcg) were moderately sensitive to the pathogen.

V. alginolyticus was resistant to the rest of the antibiotics such as Cephaloridine(30 mcg), Linomycin (2 mcg), Methicillin (5 mcg) , Oleandomycin (15 mcg), Penicillin (10 units), Tobramycin (10 mcg), Tetracycline (30 mcg), Oxytetracycline(30 ug), Tiamulin (30 ug), Bacitracin (10 ug).

Among the ten antiseptic chemicals , 6 were found to be effective in suppressing pathogen, V. alginolyticus.

Copper sulphate (0.5%), Formalin, Acriflavin (50 ppm), Methylene blue (10 ppm) were very effective whereas Potassium di chromate, (1.0 %) Malachite green (500 ppm) were found to be less effective.

A new product Virkon which is a (disinfectant) was found to be effective at 0.5 % level. This compound drastically reduced the growth of V. alginolyticus in broth cultures.

The results obtained from natural products (bioactive compounds) suggested the possibilities of using sponge extracts for controlling V. alginolyticus.

REFERENCES

ANDERSON,J.I.W. and CONROY, D.A. 1970
Vibrio diseases in Marine Fishes. In S.F.Snieszko (Editor), A symposium on Diseases of Fishes and Shellfishes. Am. Fish. Soc., Washington, Dc, spec. Publ. No.5, 266-272.

ANDERSON,I.G, SHAMSUDIN,M., SHARIFF and NASH,G. 1988
Bacterial septicemia in juvenile tiger shrimp, *P. monodon*, cultured in Malaysian brackish water ponds. Asain Fisheries Science, 2: 93-108.

AUSTIN,B., MORGAN, D.A. and ALDERMAN, D.J. 1981/82
Comparison of antimicrobial agents for control of vibriosis in marine fish. AQUACULTURE 26(1).

AUSTIN,B., JOHNSON, C. and ALDERMAN, D.J. 1982
Evaluation of substituted quinolines for the control of Vibriosis in turbot (*Scophthalmus maximus*)
AQUACULTURE 29: 227

AUSTIN,B; DAY,J.G. 1990
Inhibition of prawn pathogenic *Vibrio* spp. by a commercial spray-dried preparation of *Tetraselmis suecica*.Dep.Biol.Sci. AQUACULTURE, 90 (3-4) 389-92.

AVELIN,Sr., RAMILA,G. & Sr, VITALINA 1991
Effect of the red alga *Hypnea musiformis* extract on the humoral immune responses in *oreochromis mossambicus* (Peters). Bull. Seaweed. Res. Utiln. 14(1): 25-85.

BATICADOS,M.C.L; LAVILLA - PITOGO,C.R; CRUZ-LACIERDA 1990
Studies on the chemical control of luminous bacteria *Vibrio harveyi* and *V. splendidus* isolated from diseased *Penaeus monodon* larvae and rearing water. DIS. = AQUAT.-ORG, 9(2): 133-139.

BAUER,A.W., KIRBY,W.M.M., SHERRIS,J.C. 1966
Antibiotic susceptibility testing by a standarized single disk method. Am.J. Clin.Pathol., 45(4): 493 - 496

BELL,T.A. 1992

Drugs and chemotherapentants for shrimp diseases: Their present status in the US, with an overview of Research and approval process. In Diseases of cultured Penaeid shrimp in Asia and the US. (Edn. Fulks and K.L.Main). Oceanic Int. Hawaii. pp.311-324.

BELL,T.A.; LIGHTNER, D.V. 1987

An outline of Penaeid shrimp culture methods including infectious disease problems and priority drug treatments. VET. - HUM - TOXICOL, 29 (1): 37-43.

BRICK, R.W.

Effects of water quality, antibiotics, phytoplankton and food on survival and development of larvae of *Scylla serrata* (Crustacea : Portunidae) AQUACULTURE 3(2): 231 - 244.

BROWN, J.H. 1989

Antibiotics: Their use and abuse in aquaculture. Inst. Aquacult., Univ. Stirling, UK. WORLD - AQUACULT, 20(2):34-43.

CARL, J. SINDERMANN. 1990

Principal diseases of Marine Fish and Shellfish, 2:57-58.

COLLINS,C.H., PATRICIA M. LYNE, GRANGE,J.M. 1989
Antimicrobial sensitivity and assay tests. Microbiological methods. 6th Edition. pp. 155-167.

COLORNI,A. 1985
A practical method for determining the susceptibility of aquatic Vibrio and Aeromonas spp. to aantibacterial agents. AQUACULTURE, 46: 263

CORLISS,J.P. 1979
Accumulation and depletion of Oxytetracycline in juvenile white shrimp *Penaeus setiferous*. AQUACULTURE, 16(1): 1-6.

EGIDIUS,E., 1987
Vibriosis: Pathogenicity and pathogen. A review. AQUACULTURE, 67: 15-28.

EGUSA,S.Y., TAKAHASHI,T., ITAMI and MOMOYAMA,K. 1988
Histopathology of Vibriosis in the Kuruma prawn, *Penaeus japanicus*. Bate. Fish Pathol., 23: 59-65

ERICSSON,H.M. and SHERRIS,J.C. 1971
Antibiotic sensitivity testing. Report of an International collaborative study. Acta Pathologia Microbiologia Scandinavica, Supplement No.217, Section B.

GERUNDO,N., ALDERMAN,D.J. 1991
Pathological effects of repeated doses of Malachite green : a preliminary study. J. FISH DISEASES, 14: 521 - 532.

HAMEED,- A.S.S.; RAO,-P.V. 1994
Studies on the chemical control of a *Vibrio campbelli* - like bacterium affecting hathery reared *Penaeus indicus* larvae. AQUACULTURE, 127(1): 1-9.

HAMEED,A. SHAHUL 1989
Studies on the pathobiology of Penaeid larvae and post-larvae. Ph.D thesis, Cochin Univ. of Sciences and Technol., Cochin, India. pp. 243

HUSEVAG,B. and LUNESTAD, B.T. 1991
Simultaneous occurrence of *Vibrio salmonicida* and antibiotic - resistant bacteria in sediments at abandoned aquaculture sites. J. FISH DISEASES, 14: 631 - 640

ITAMY.T, TAKAHASHI.Y, NAKAMURA.Y. 1989
Efficacy of vaccination against Vibriosis in cultured prawns *P. japonicus*: J.AQUAT. ANIM. HEALTH, 1(3): 238 - 242

ITAMI.T; TAKAHASHI.Y; YONEOK.K. 1991
Survival of larval giant tiger prawns *P. monpdon* after addition of killed *Vibrio* cells to a microencapsulated diet. J.AQUAT.- ANIM.- HEALTH., 3(2): 151 - 152

ITAMY.T, YAN.Y, TAKAHASHI.Y. 1992
Studies on vaccination against Vibriosis in cultured Kuruma prawn *Penaeus japonicus*. : Effect of different vaccine preparations and oral vaccination efficiency. J. SHIMONOSEKII - UNIV.-FISH. - SUISAND - AI - KENPO, 40(3): 139 - 144

LAVILLA - PITOGO,C.R., BATICADOS,M.C.L., CRUZ-LACIERDA,E.R. 1990
Occurrence of luminous bacterial disease of *Penaeus monodon* larvae in the Philippines. Aquaculture, 91: 1-13

LEWIS,D.H. 1973
Response of brown shrimp to infection with *Vibrio sp.* Proc. Annu. Meet. World Maric. Soc., 4: 333-338

LIGHTNER,D.V., 1983

Diseases of cultured Penaeid shrimp. In. CRC Hand book of mariculture. Crustacean Aquaculture. (J.P.MC Vey, ed.), pp. 289-320. CRC Press, Boca Raton, Florida.

LIGHTNER,D.V., REDMAN,R.M., DONALD,D.A., WILLIAMS,R.R., and PEREZ,L.A. 1984

Major diseases encountered in controlled environment culture of penaeid shrimp at Puerto Penasco, Sonora, Mexico. In Proceedings of the ninth and tenth US - Japan Meetings on Aquaculture. (C.J.Sindermann, ed.), NOAA Tech. Rep. NMFA 16, pp. 25-33. US. Dep. Commerce, Washington, D.C.

LIGHTNER.D.V. 1988

Vibrio disease of Penaeid shrimp. In Disease diagnosis and control in North American Aquaculture, second revised edition. (Eds. C.J.Sindermann and D.V.Lightner)

Elsevier, Amsterdam pp. 42 - 47

LIGHTNER,D.V., BELL,T.A., REDMAN,R.B., MOHNEY,L.L. NATIVIDAD,J.M., RUKYAMI,A. and POERNOMA,A. 1992
A review of some major diseases of economic significance in penaeid prawns/shrimps in the Americas and Indo Pacific. In M. Shariff, R.P.Subasinghe and J.R.Arthur (Eds.). Diseases in Asian Aquaculture I. Fish Health section, Asian Fisheries Society, Manila, Philippines. pp. 57-80.

LIMSUWAN, C 1988
Causative agent and control of black splint disease in *Penaeus monodon*. Proceedings of the seminar on shrimp diseases and their control. 7-8 Dec. Natural Inland Fisheries Institute Bangkok, Thailand.

LIPTON,A.P. 1994
Shrimp diseases and control. Manual. Workshop on Shrimp Farming Techniques, Problems and Solutions. M.K.Univ. pp. 34-45.

MEYER,F. 1991
Aquaculture disease and health management. J. Anim. Sci., 69: 4201-4208.

MC CARTHY, D.H., STEVENSON,J.P. And SALSBURY,A.W.
Combined in-vitro activity of trimethoprim and sulphonamides on fish pathogenic bacteria. AQUACULTURE, 3(1): 87-91.

PADMINI SREENIVASA RAO,P. SREENIVASA RAO,P. and KARMARKAR,S.M. 1990
Biological investigation of Indian phaeophyceae. 8. Antimicrobial activity of *Sargassum wightii*. Greville Bull. Seaweed Res. Utiln., 12 (1 & 2): 577-582.

RAMA RAO,K. 1982
Biology of *Hypnea* species and their possible utilisation in India. Bull. Seaweed Res. Utiln., 5: 11-19.

ROSILY, N.P.; SREEKUMARI, K.R.; SHARMA, A.V. 1987
Drug resistant *Vibrio sp.* associated with larvae of prawn *Penaeus indicus*. Proceedings of the national seminar on Estuarine Management, TRIVANDRUM, pp. 477-480.

RUANGPAN,L. 1982
Diseases and parasites of *Penaeus monodon* fabricious. Thai. Fish. Gaz., 35: 385-387.

RUANGPAN, L. and KITAO,T. 1992
Minimal inhibitary concentration of 19 chemotherapeutants against *Vibrio* bacteria of shrimp, *Penaeus monodon*. In Diseases in Asian Aquaculture (ed. by M. Shariff, R. Subasinghe & J.R.Arthur), pp. 135-142. Asain Fisheries Society, Manila.

RUANGPAN,L. and SAE-VID, 1988
Melanosis in black tiger prawn, *P. monodon*. Proceedings of the seminar on shrimp diseases and their control, 7 - 8, Dec. National Inland Fisheries Institute Bangkok, Thailand.

SANGRUNGRUANG, K; AOKI, T. ; YOSHIDA, T. 1993
Minimal inhibitory concentrations of II chemotherapeutants and drug resistance against *Vibrio* bacteria isolated from shrimp (*Penaeus monodon*) in Thailand. NRCT - JSPS Joint seminar on marine science, Songkhla (Thailand), 2-3 Dec.

SANO,T. and FUKUDA,H. 1987
Principal microbial diseases of mariculture in Japan. AQUACULTURE, 67: 59-69.

SCHAPERCLAUS,W. 1991
Fish diseases, 1: 215

SHAHUL HAMEED,A.S. 1994
Reduction in chaetoceros population growth by Antimicrobials. Asian Fisheries Science, 7: 273-275.

SHAHUL HAMEED, A.S., RAO,P.V. 1994
Studies on the chemical control of a *Vibrio campbellii* like bacterium affecting hatchery - reared *Penaeus indicus* larvae. Aquaculture, 127: 1-9.

STOKES,E.J., and RIDGWAY,G.L. 1987
Clinical Microbiology, 6th Edn. Edward arnold, London, pp. 213, 236.

SUNARYANTO,A. and MARIAM,A. 1986
Occurrence of a pathogenic bacteria causing luminescence in penaeid larvae in Indonesian hatcheries. Bull. Brackishwater Aqua. Devl. Centre, 8: 64-70.

SUNG,H.H., KOU,G.H. and SONG,Y.L. 1994
Vibriosis Resistance induced by Glucan Treatment in Tiger shrimp (*Penaeus monodon*). J. FISH PATHOLOGY, 29 (1): 11-17.

TAKAHASHI,Y., SHIMOYAMA,Y. and MOMOYAMA.K, 1985 a
Pathogenicity and characteristics of *Vibrio sp.* isolated from diseased post-larvae of Kuruma prawn, *Penaeus japonicus* Bate. Bull. Jap. Soc. Sci. Fish, 51: 721-730.

TAKAHASHI,Y. ITAMI,T., NAKAGAWA,A., NISHIMURA,H. and ABE,T. 1985 b
Therapeutic effects of oxytetracycline trial tablets against Vibriosis in cultured kuruma prawn *Penaeus japonicus*. Bate. Bull. Jap. Soc. Sci. Fish, 51: 1639-1643.

TANSUTAPANIT,D.S.O.A, and RUANGPAN,L. 1987
V. harveyi a causative agent of white shrimp nauplii, *Penaeus merguiensis*. In Abstract. Tech. paper No.6/30. Third Natl. Seminar on Marine Science, 6-8. August 1986, NSRC, Bangkok, Thailand.

VANDERZANT,C. MROZ,E. and NICKELSON,R. 1970
Microbial flora of Gulf of Mexico and pond shrimp. J. Milk Food Technol., 33: 346-350.

WENDY FULKS and KEVAN L. MAIN, 1992
Diseases of cultured Penaeid shrimp in Asia and the United States. Proceedings of a workshop in Honolulu, Hawaii, pp 40-41, 197-200, 349-352. Pub.by: The Oceanic Institute, Makapuu point P.O. Box 25280, Honolulu, Hawaii, 96825.