STUDIES ON INDUCED MATURATION, SPAWNING AND LARVAL SETTLEMENT IN GREEN MUSSEL *Perna viridis* (LINNAEUS, 1758)

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DEDICATED

TO

MY BELOVED PARENTS

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CERTIFICATE

Certified that the thesis entitled "**STUDIES ON INDUCED MATURATION, SPAWNING AND LARVAL SETTLEMENT IN GREEN MUSSEL *PERNA VIRIDIS* (LINNAEUS, 1758)**" is a record of independent bonafide research work carried out by **Mr. Manoj Nair. R** during the period of study from November 1996 to July 2001 under our supervision and guidance for the degree of **Doctor of Philosophy (Mariculture)** and that the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title.

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I hereby declare that this thesis entitled "**STUDIES ON INDUCED MATURATION, SPAWNING AND LARVAL SETTLEMENT IN GREEN MUSSEL *PERNA VIRIDIS* (LINNAEUS, 1758)**" is an authentic record of the work done by me and that no part there of has been presented for the award of any degree, diploma, associateship, fellowship or other similar title.

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सारांश

हरित शंबु *पेर्ना विरिडिस* (लिनेयस, 1758) के प्रेरित परिपक्वन, अंडजनन, डिंभक पालन एवं जमाव पर अध्ययन किए गए. *पेर्ना विरिडिस* को 23 +-1°C तापमान, > 30 पी पी टी लवणता, 7.5 -8.2 पी एच में रखा गया और कीटोसिरस कालिभिद्रन्स 1x106 मि लि कोश सान्द्रता तथा 700 आइ.यू. कोड लिवर ओइल से संपुष्ट करके प्रतिदिन प्रति जीव को एक लिटर की दर में 15 तथा 32 दिनों के अंतराल में खिलाने पर इसके ब्रूड स्टॉक को पूरे अंडजनन काल में परिपक्वन के लिए प्रेरित किया जा सकता है. >25 पी पी टी लवणता तथा 7 - 8.2 पी एच में जल के परिवेश ताप से 5°C बढ़ाकर इस में नर या मादा *पी. विरिडिस* के गैमीट को डालने पर 1- 2 घंटे में ही अंडजनन के लिए प्रेरित किया जा सकता है. कोई लिंगवार प्रतिरूप नहीं दिखाया पडा. सामान्यतः उपयुक्त किए जाने वाले रसायनों जैसे हाइड्रजन पेरोक्साइड या सेरटोनिन से अंडजनन की प्रेरणा नहीं व्यक्त हुई.

पी. विरिडिस के डिंभक पालन में भौतिक-रासायनिक प्राचलों का अध्ययन किए जाने पर मालूम पडा कि डिंभकों की अनुकूलतम बढ़ती , अतिजीविता और स्पैटों के जमाव के लिए 29° C - 31°C तापमान 50 पी पी एम क्लोराम्फेनिकोल प्रतिजैविकी के साथ > 30 पी पी टी की उच्चतम लवणता तथा 8.2 पी एच, साधारण वातन और खाद्य के रूप में आइसोक्राइसिस गालबाना देना उचित देखा गया. डिंभक जमाव के प्रारंभ में तंतुल आधार पसंद करते हैं और प्रौढ़ शंबुओं का आकर्षण होने पर भी इन आधारों में बाइसस सूत्र अच्छे देखे गए. डिंभकों के अच्छे जमाव के लिए उच्च तापमान 31°C, साधारण वातन, 25-38 पी पी टी लवणता , 7-8 पी एच अनुकूलतम देखे गए. अच्छे जमाव के लिए उपयुक्त विभिन्न रसायनों में *L - DOPA* देने पर उत्कृष्ट परिणाम निकला कि $2.5x10^{-7}$ M में 5 घंटों में 100 % जमाव हुआ. परिवेश आर्द्रता की स्थिति में परिवहन करने पर शंबुओं का सुदूर जमाव सफल हुआ. 24 घंटे के परिवहन से *पी. विरिडिस* के डिंभकों के जमाव पर कोई असर नहीं पडा है.

ABSTRACT

In the green mussel *Perna viridis* (Linnaeus, 1758), studies were undertaken on the induced maturation, spawning, larval rearing and settlement.

Perna viridis broodstock could be conditioned or induced to mature out of the spawning season by maintaining them at 23 ± 1 ° C, at > 30 ppt salinity, pH 7.5 – 8.2 and fed with *Chaetoceros calcitrans* at a cell concentration of $1x\ 10^{6}\ ml^{-1}$ fortified with 700 I.U. cod liver oil @ $1\ l\ animal^{-1}\ day^{-1}$ in two installments for 15 and 32 days respectively.

An increase of 5 ° C form the ambient water temperature at a salinity > 25 ppt and pH 7 – 8.2 with an addition of male or female gametes was able to induce 100 % spawning in 1 –2 hours in *P. viridis*. No sex wise pattern was observed. None of the commonly used chemicals like Hydrogen peroxide or Serotonin was able to elicit a full spawning response.

Studies on the effect of Physico – chemical parameters for larval rearing of *P. viridis* showed that a temperature of 29 ° C – 31 ° C at high salinity > 30 ppt with 50 ppm Chloramphenicol antibiotic at pH 8.2, with moderate aeration and fed with *Isochrysis galbana*, as the best for optimum growth, percentage survival and settlement of spat.

Settlement preference indicated clearly that primary settling larvae required filamentous substrates and among these byssus threads was the best substrate even though presence of adult mussels significantly attracted more mussels than byssal threads. High temperature 31 ° C, moderate aeration, salinity of 25 – 38 ppt, pH 7 – 8 was optimum for larval settlement.

Among the various chemicals tried L - DOPA gave the best results with 100 % settlement observed in 5 hours at 2.5×10^{-7} M.

Remote setting was also successfully done with larvae transported in ambient moist condition. A 24 hour transportation period did not significantly affect the percentage of *P. viridis* larval survival or settlement.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

GENERAL INTRODUCTION

GENERAL INTRODUCTION

Aquaculture can be defined as the act of growing aquatic organisms and harvesting the production for human benefits from coastal waters. An important branch of aquaculture is mariculture. Mariculture, the organized culture of marine organisms in the sea has a very long history. It is believed that the first attempted mariculture was that of molluscs. Japanese farmed oysters on intertidal stretches of the shore around 2000 B.C. Aristotle mentions the cultivation of oysters in Greece while Pliny gave details of Roman oyster farming from 100 B.C.

As projected by FAO in 1998 the per capita consumption of fish would be 16.1 Kg and this can be achieved only through aquaculture as less than half of the fish consumed is likely to originate from capture fisheries and mariculture will account for a large share of total production particularly with the development of viable offshore culture technologies.

The total world Fish and Shellfish aquaculture production in 1999 was provisionally put at 32.9 million metric tonnes (mmt) an increase of 2 mmt from 1998. In 1998 fish and shellfish through maricultute accounted for 35 % of the total aquacultue production with the molluscs contributing 9143 thousand tonnes, (47 %) valuing US $ 8479 million (FAO, 2000). Total food fish supply is growing at a rate of 3 – 6% per annum since 1961 and protein derived from fish, crustaceans and molluscs account for between 13.5 – 16.5 % of animal protein intake of the human population.

Molluscan species contributing to aquaculture include oysters, mussels, scallops, gastropods and clams. Marine mussels form one of the

most dominant cultivable species all around the world. Though mussels are found distributed throughout the world and are amongst the hardiest and most early gathered seafood organisms, they give the highest conversion of primary producers to human food and culture of mussels in the water column can increase seafood production several folds. Mussel culture also has an interesting accidental origin. It was believed to have been started in France by a shipwrecked Irishman Mr. Patrick Walton (Korringa, 1976). It was only post 1960 that culture began in South East Asian countries firstly on an experimental scale and later on commercial lines. The mussel as a human food is extremely nutritious and can be readily processed into high quality foods. Mussel meat is tender, easily digestible and therefore it could be the cheapest and most nutritious shellfish meat in the world market. The advantages of mussel as a candidate species for aquaculture in addition to the above is 1. It is low in the food chain, 2. The unique feature of attachment with byssal threads to the substratum, 3. Farming techniques are simple and 4. Faster growth rate.

Unlike most other aquatic species wild mussel production is much lower than cultured production. This increase is mainly via culture. Among the molluscs being cultured in 1996, mussels contributed 1.19 mmt valued at U.S. $ 0.49 thousand million. There are 12 species of mussels being cultured world wide with the bulk of the production being contributed by the Blue mussel *Mytilus edulis*, Mediterranean mussel *Mytilus galloprovincialis*, Green mussel *Mytilus smaragdinus*, New Zealand mussel (green lip or green shell) *Perna canaliculus*. The main countries involved in mussel culture are China, Thailand, Singapore and Philippines in Asia, Spain, Italy, Netherlands and France in Europe, New Zealand and Australia in the Oceanica.

India has two commercially important species of mussels. Green mussel *Perna viridis* and the Brown mussel *Perna indica*. In Indian literature

green mussel was often described as *Mytilus viridis* and Kuriakose and Nair (1976) has synonymised this under *Perna viridis*. The former has a wide distribution along the Indian coasts and about 10,000 tonnes of mussels out of a potential of 22080 tonnes are exploited yearly from the west coast of India mainly from the state of Kerala (Appukuttan *et al.*, 2001). They support traditional sustenance fishing and scope of increasing natural production from existing beds is rather limited.

Mussel culture in India using *Perna viridis* and *Perna indica* was first conducted on an experimental scale in the early seventies by the Central Marine fisheries Research Institute (C.M.F.R.I) at Calicut and Vizhinjam. Though the technology was developed by CMFRI in the mid and late seventies, it was only two decades later that the first commercial culture of mussels (Green mussel *Perna viridis*) was started in late 1995 at Anthakaranazhi (Alleppey District) in Kerala by local fishermen on long lines in the sea with the technical support of CMFRI. Commercial mussel culture activity along the south west coast of India picked up in a big way since 1997 in different parts of Kerala and Karnataka (Mohamad *et al.*, 1998; Velayudhan *et al.*, 2000) and cultured mussel production is expected to be about 1000 tonnes in the year 2000 and the production is projected to be 120,00 tonnes by the year 2007 – 2008 (Appukuttan, Personal Communication).

At present seed requirement for mussel farming in India is met mainly from the wild. It is observed that profuse mussel spat settlement occurs in the intertidal and subtidal areas during the post monsoon period. However, a substantial quantity of these spat perish due to various adverse ecological conditions (Appukuttan *et al.*, 2001). As mussel farming expands, the farmers cannot solely depend on the seeds from the wild because there is a tendency in recent years for poor settlement due to a failure in spawning or some other

unforeseen natural factors. Moreover, the indiscriminate seed collection from the wild alone would lead to social problems with the traditional mussel pickers. Hence the non-availability of sufficient quantities of seed would become the major bottleneck in the expansion of mussel culture in India. Recent studies indicate that the seed availability for farming is limited and the seed requirement cannot be met only from the wild for commercial scale mussel production by culture (Appukuttan *et al.*, 2001). Thus the development of a commercially viable hatchery technology package becomes a priority for a long term sustainable and successful mussel farming in India.

Hatchery technology of Indian mussels *Perna viridis* and *Perna indica* has been attempted in the late seventies and early eighties (Rao *et al.*, 1976; Rengarajan 1983 *a*; Appukuttan *et al.*, 1984, 1988; Sreenivasan *et al.*, 1988 *a, b,* 1989 *a*). Commercial cost effective technology for hatchery production of seeds could not be sustained thereafter due to various factors.

For any successful hatchery technology the most important aspect is the complete control of the life cycle of the candidate species under captivity. The first step in this direction is to induce maturation of the animals. In bivalve molluscs induced maturation studies have been successfully carried out by scientists of CMFRI in the pearl oyster *Pinctada fucata* and the edible oyster *Crassostrea madrasensis* (Alagarswami *et al.*, 1987; Nayar *et al.*, 1987). No attempt has been made to study these factors in commercially important mussels.

A successful spawning protocol with maximum yields of viable gametes is also very important in the development of a full-fledged hatchery package. Though several methods have been used to induce spawning of bivalves in India (Alagarswami *et al.*, 1983, 1987; Nayar *et al.*, 1987), only

preliminary attempts were made in the mussel *Mytilus viridis* by Rao *et al.* (1976).

Larval rearing is also very important for the success of any hatchery. As the amount of larvae, which ultimately settle to spat in numbers, is very crucial for it's financial viability. For optimization of the conditions for healthy larval growth, it is necessary to know the various factors, which influence larval life stages in controlled hatchery conditions thus helping to increase the growth, survival and yield of the larval stock. Some initial experiments on larval development and life cycle in *M. viridis* and *Perna viridis* has been conducted (Rao *et al.*, 1976; Rengarajan, 1983 *a*; Sreenivasan *et al.*, 1988 *a*, *b*, 1989 *a*).

In the hatchery cycle the "settlement of bivalve molluscan larvae from a pelagic swimming eyed to a creeping pediveliger larvae and the final spat is a crucial phase". Many workers have reviewed settlement of marine invertebrates including molluscs. Several physical, chemical and biological cues have been identified as factors which enhance settlement in these animals (Crisp, 1974; Bayne, 1976; Bonar *et al.*, 1990; Pawlik, 1992; Rodriguez *et al.*, 1993).

Though some preliminary experiments on settlement of spat was conducted in *M. viridis, Perna viridis* and *Perna indica* (Rao *et al.*, 1976; Rengarajan, 1983 *a*; Appukuttan *et al.*, 1984, 1988), no attempt has yet been made to study the various factors which influence settlement in the green mussel *Perna viridis*.

The present study is designed to probe into the details of the basic aspects of the biology related to the hatchery technology of *Perna viridis* and the understanding of the factors which influence the inducement of maturation, spawning, larval rearing and spat settlement which would go a long way in the

upgradation of hatchery technology of *Perna viridis* for a commercial level seed production.

This study consists of four chapters briefly explained below,

Each chapter contains an introduction, which also contains the review of literature, materials and methods, results and discussion.

The first chapter deals with the methods to try to condition and induce maturation in captivity in *Perna viridis* using a) different feeds, b) under different salinity and c) different pH at 2 different temperatures 23 ° C and 31 ° C (Ambient).

The second chapter studies factors, which induce spawning in *P. viridis* in captivity using different physical, chemical and biological methods. Here spawning was induced by different salinity, pH, thermal stimulation, desiccation; chemicals like Tris, Hydrogen peroxide and using sperm and egg suspension.

The third chapter looks in to the optimum physico chemical parameters required for *P. viridis* larval rearing. Here the effect of salinity, pH, temperature, aeration, antibiotics, feed on *P. viridis* larvae was studied.

The fourth and the last chapter deals with the factors which influence *P. viridis* larval settlement. Here effect of substrates, seaweed, adult mussel, light, colour, temperature, aeration, salinity, pH, chemicals like L – DOPA, GABA, Serotonin, Potassium, Ammonia, Copper on *P. viridis* larval settlement was looked into. Remote setting of *P. viridis* larvae was also tried.

CHAPTER I

INDUCED MATURATION OF *PERNA VIRIDIS*

1. INTRODUCTION

The timing of reproduction in a multi species population of invertebrates takes many patterns. Continuous reproduction has been reported in many tropical species. Reproduction in continuously breeding tropical species is not likely to be of the same intensity throughout the year, however, when closely examined several populations show periods of intense reproduction (Giese and Pearse, 1974). A breeding season usually lasts for one month or so followed by a period of another month or more of almost no reproductive activity in almost all the individuals. Many tropical species including mussels have a bi or semi annual breeding season which is characteristic of areas influenced by monsoons (Paul, 1942; Antony Raja, 1963). Giese and Pearse (1974) have reviewed some of the factors that regulate the course of gonad development to maturation in bivalve molluscs.

Reproductive cycles of mussels and other marine bivalves are affected by interactions of endogenous (nutrient reserves, hormonal cycles and genotype) and exogenous factors (temperature, salinity, light and food) (Giese and Pearse, 1974; Sastry, 1979). The environmental factors responsible for bringing a population to a mature stage so that spawning can be coordinated thereby synchronizing the release of gametes have not received much

attention. Although some of these factors affecting the reproduction of mussels have been investigated experimentally, most information has been through field observations (Seed, 1976; Seed and Suchanek, 1992).

Seasonal gonad developments of molluscs restrict the spawning to a particular seasons of the year. Molluscs spawn naturally during certain seasons in a year when the environmental conditions are congenial for this activity. This physiological characteristic generally limits the process of seed production and availability to certain particular periods, which is highly disadvantageous in the commercial culture of molluscan species. In this context, the concept of induced maturation gains importance as the process can be advantageously controlled for a prolonged period of seed production. For any successful hatchery technology, the most important aspect is the complete control of the life cycle of the candidate species under captivity. The first step in this direction is the successful induction of maturity in captivity. In the present study induced maturation refers to the accelerated gonadal development using different techniques to achieve sexual maturity, so that they can be used for seed production even when they are comparatively young and out of the spawning season.

The term conditioning has been used in literature in tandem with induced maturation and is a little confusing. Several of the early works and some recent ones have used this term for induced maturation experiments. The various techniques of hatchery conditioning of broodstock of bivalves have been reviewed recently

(Utting, 1993, Utting and Millican, 1997, 1998). In Mussels, gonad conditioning has been achieved in *Mytilus edulis* (Bayne, 1965; Hrs– Brenko, 1973 *b;* Sprung, 1984 *a – d*), *M. viridis* (AQUACOP, 1979), *Perna perna* (Siddall, 1980) and *Perna viridis* (Siddall, 1980; Sreenivasan *et al.*, 1988 *a*).

The technique of maturation and spawning of bivalve molluscs out of season was revolutionized by Loosanoff and Davis (1950, 1952, 1963) in *Venus mercenaria.* In mussels it has been done mainly in *M. edulis* (Bayne, 1965, 1972; Bayne *et al.*, 1975, 1978, 1982)

The role of salinity in initiation of gametogenesis in captivity has been studied in mussels (Wilson, 1968, 1969; Bayne *et al.*, 1978).

2. MATERIAL AND METHODS

Geographical description of the collection and study areas (PLATE I A) are given below.

Cochin (9 ° 59 ' N latitude and 76 ° E longitude) is situated on the south west coast of India in Kerala State.

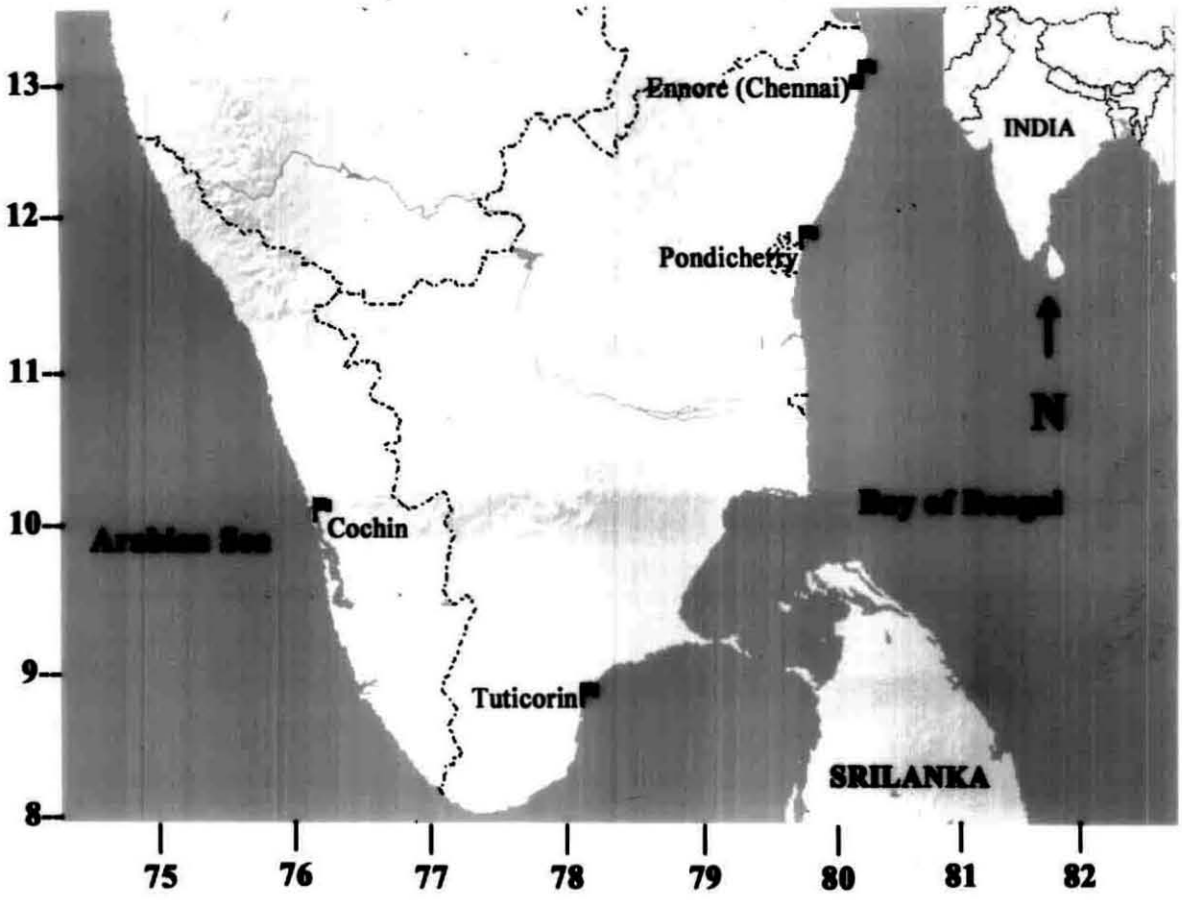
Tuticorin Research Centre (TRC) of C.M.F.R.I. (8 ° 45 ' N latitude and 78 ° 12 ' E longitude) is located in Tamil Nadu on the south east coast of India.

Pondicherry (11° 46 '- 12 ° 30 ' N latitude and 79 ° 36 ' – 79 ° 53 ' E longitude) is situated on the south east coast of India nearly 160 km south of Chennai.

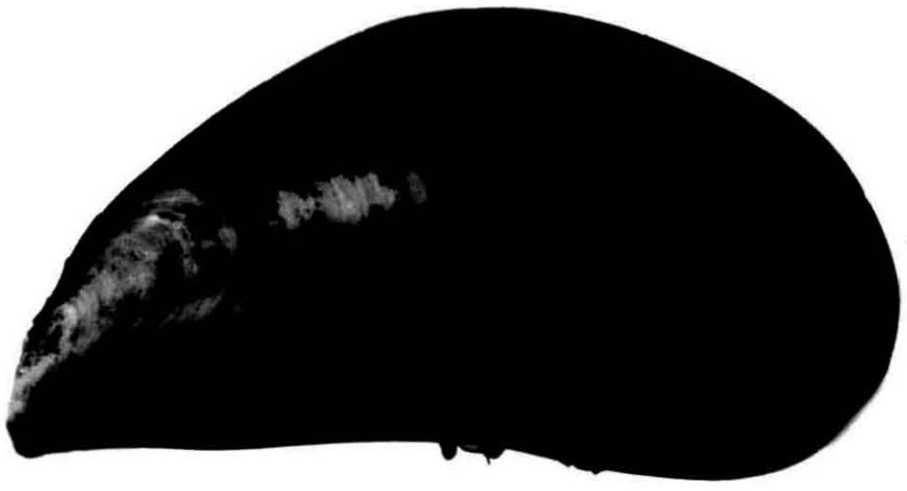
Ennore (13 ° 14 ' N latitude – 80 ° 20' E longitude), is located 20 Km north of Chennai, Tamil Nadu on the south east coast of India.

Specimens of Green mussel *Perna viridis* (Linnaeus, 1758) (PLATE I B), were collected from Ennore and Pondicherry. All the experiments of induced maturation, spawning, larval rearing and settlement were conducted in the shellfish hatchery at Tuticorin Research Centre of C.M.F.R.I, Tuticorin. Only a part of the remote setting experiment was conducted at the hatchery complex, Head Quarters, Cochin.

PLATE I



A. Collection areas of ***Perna viridis***



B. ***Perna viridis*** (Linnaeus, 1758)

2.1.Maintenance of sea water quality

Sea water for all the experiments was pumped into a settling tank and then by gravity into a biological filter bed and passed through a rapid sand filter. Filtered seawater from here was stored in a concrete sump from where it was distributed to all parts of the hatchery by PVC pipes.

2. 2. Water quality parameter estimations

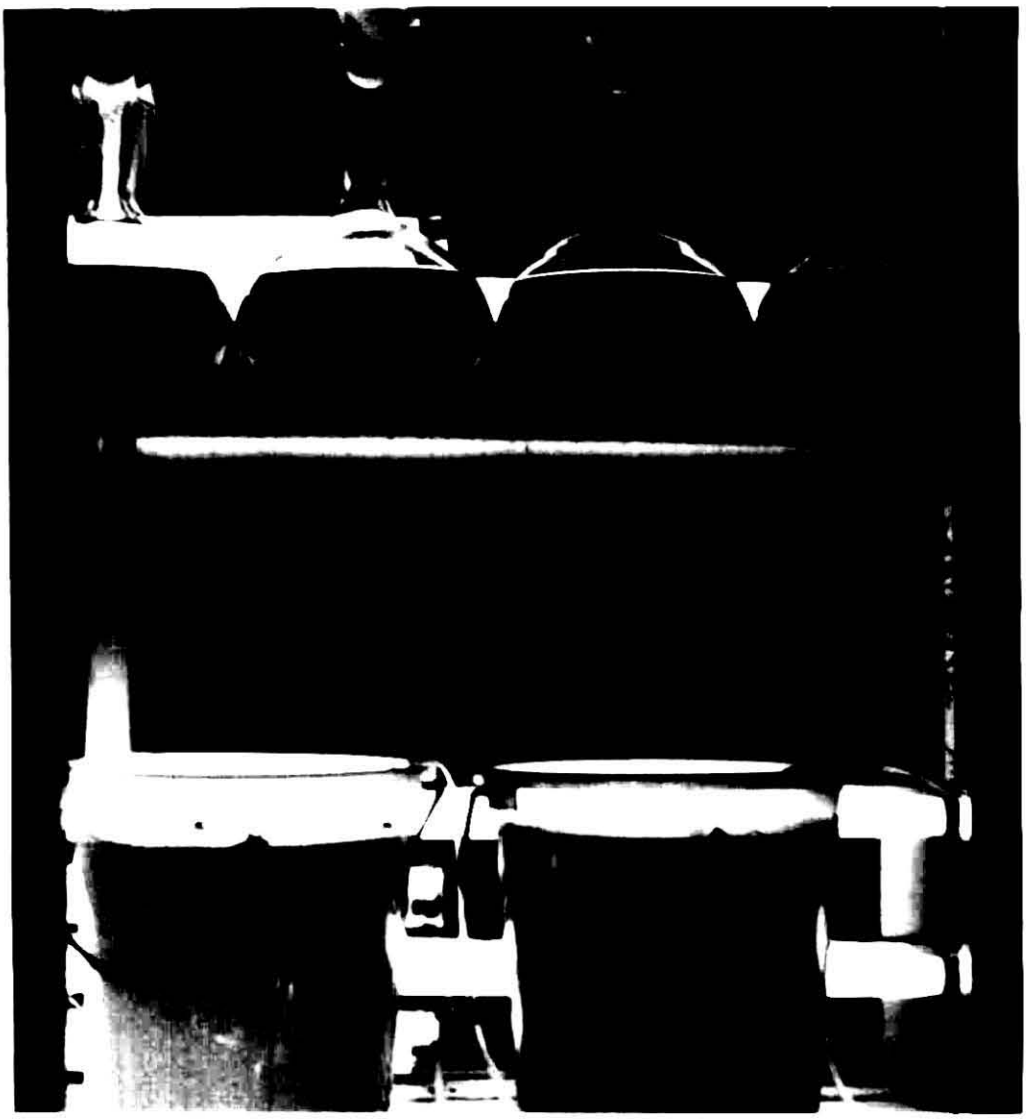
Water quality parameters like salinity, pH, Dissolved Oxygen, Ammonia, Hydrogen Sulfide were estimated according to Strickland and Parsons (1972).

The water quality parameters were monitored daily. For the conditioning experiments, the different parameters considered were air temperature: 33 ± 1 ° C (23 ± 1 ° C in A.C. room), water temperature: 29 ± 1° C (23 ± 1 ° C in A.C. room), salinity: 35 ± 1 ppt, pH : 8.0 ± 0.1, Dissolved Oxygen : 4.8 ± 0.3 ppm, Ammonia : 0.001 ppm and H_2 S nil. Whereas for induced maturation, spawning experiments the parameters were air temperature: 35 ± 1° C (23 ± 1 ° C in A.C. room), water temperature: 31 ± 1° C (23 ± 1 ° C in A.C.room), Salinity : 38 ±1 ppt, pH : 8.2 ± 0.1, Dissolved Oxygen : 5.2 ± 0.3 ppm, Ammonia : 0.00136 ± 0.0001 ppm and H_2 S : nil.

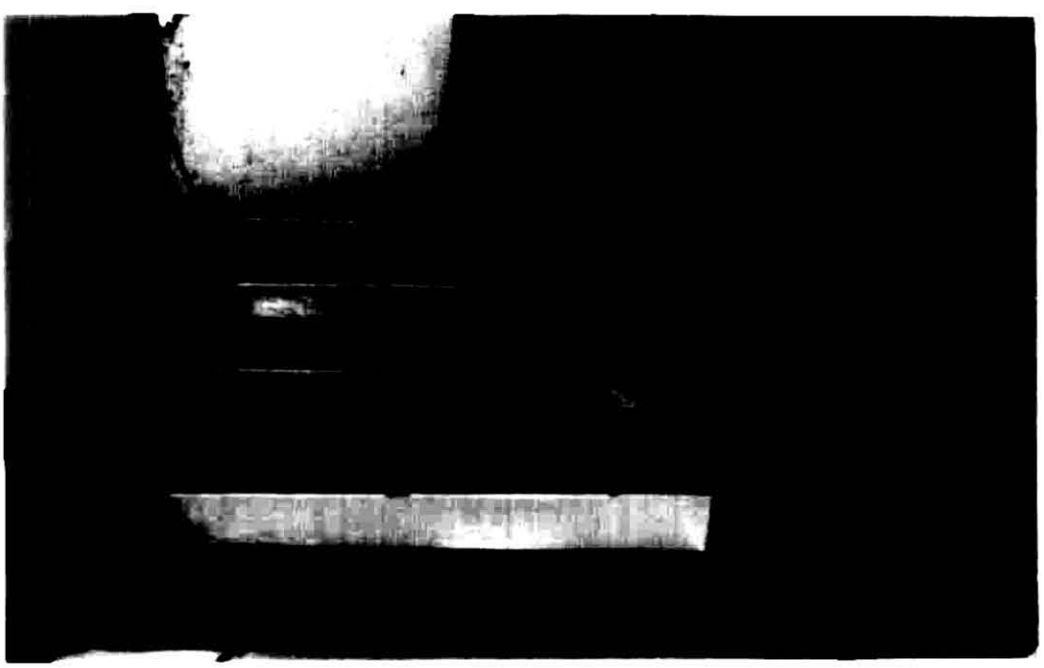
2. 3. Live microalgal feed preparation

Microalgae *Isochrysis galbana*, *Chaetoceros calcitrans*, *Nanochloropsis* sp. , *Chlorella salina* and *Spirulina* sp. were used as

PLATE II



A. Microalgal culture



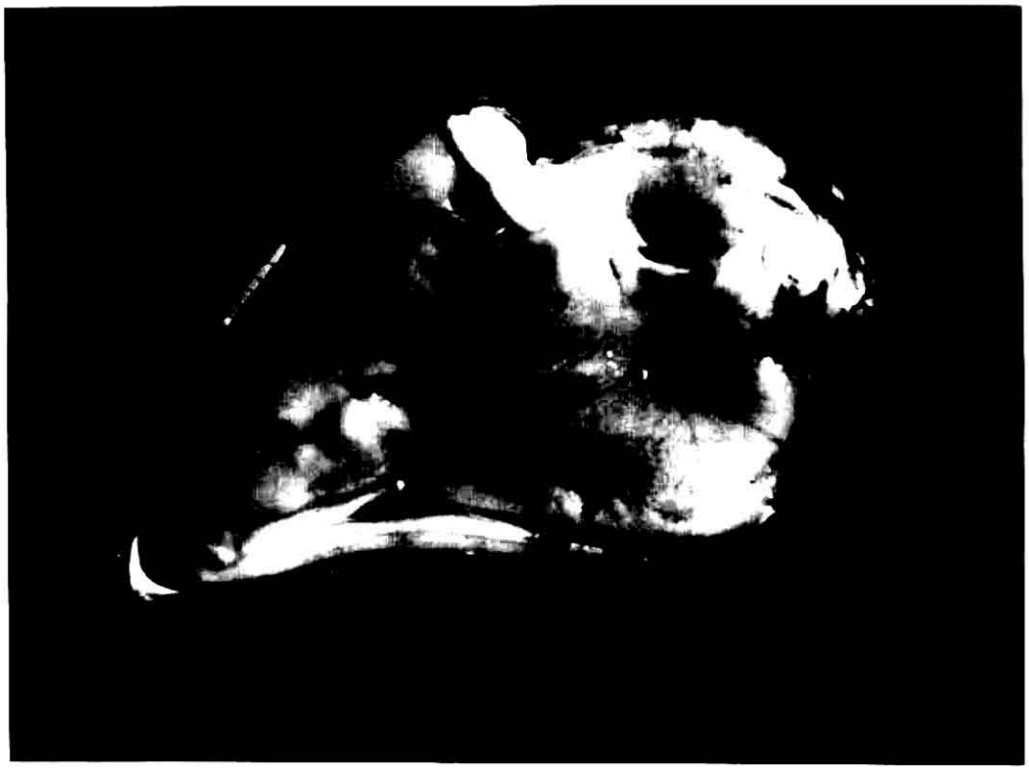
B. Condition room for induced maturation experiments

feed for the different experiments with *Perna viridis*. These algal strains were locally isolated from the bay water of Tuticorin and maintained in filtered heat sterilized sea water as pure algal stock cultures in 5 l Haffkine flasks maintained in low temperatures (24 ± 1° C) under a fluorescent lighting of 12 hour cycle (2000 lux) (PLATE II A). Depending on the need, aseptic cultures were used directly or prepared in 20 l plastic transparent buckets or 20 l glass carboys inoculated with the required exponential stage cultures aseptically. The cultures were harvested in the exponential phase and used after assessing the cell counts using a Haemocytometer. The medium used for enriching the sterilized seawater to grow all the algae was the conventional Walne's medium (Walne, 1974). Outdoor pure cultures in 20 l tubs and 1 ton white bottomed FRP tanks were also maintained for the use in conditioning and induced maturation experiments. Only exception to the above was for *Spirulina* sp. where a commercially available *Spirulina* powder (Parrys) was diluted in fresh water and fed after adjusting its concentration to the required level.

2. 4. Staging of the gonadal condition

Mussels of average length of 70 ± 5 mm were used for both the conditioning and induced maturation experiments. The male and the female gonads (30 numbers each) were staged (Stage I. Indeterminate / early maturing; Stage II. Mature; Stage III. Ripe (PLATE III A, B) and Stage IV. Spent) visually according to the scheme of Appukuttan and Nair (1983) on the basis of their colour and the area they occupied in the shell. To confirm the reproductive

PLATE III



A. Ripe male *Perna viridis*

B. Ripe female *Perna viridis*

condition, smears of the gonad and histological sections (prepared following the standard procedure of Weesner (1960) were also made and observed under low power magnification (x 10) of a compound microscope.

2. 5. Conditioning of broodstock

Conditioning experiments were carried out in Cochin from March to April 1998 and at Tuticorin shellfish hatchery of CMFRI from August to September 1998.

Thirty mussels (10 for each replication) in stage II (Mature) were maintained for each treatment in 100 l FRP basins. The treatments given were temperature 23 ± 1° C (maintained in air conditioned room) (PLATE II B) and 31 ± 1° C (ambient control temperature), two salinities 20 ppt. (made by diluting ambient salinity sea water of 35 ± 1 ppt. with fresh dechlorinized tap water) and 35 ppt. and fed on two different microalgal feeds *I. galbana* and *C. calcitrans.*

The animals were subjected to spawning by thermal stimulation on the 5 th , 10 th and 15th day after the conditioning trial started. The experiment was terminated after 15 days. The animals thereafter were sacrificed individually and the gonadal condition of each animal noted visually and tabulated as percentage mean ± standard deviation for each stage.

2. 6. Induced maturation experiments

In maturation studies, experiments were conducted for different microalgal feeds, different salinity and pH regimes on gonadal maturation. The experiment was carried out for 60 days from September to November 1999. Mussels with gonads in the stage 1 were used for this experiment. For each treatment 120 animals (40 numbers. in each replication in 200 l FRP tanks containing fresh filtered sea water) were used. In the first treatment, the effect of different microlagal feeds on maturation *viz.* pure cultures of *C. calcitrans*, *I. galbana*, *Chlorella salina*, *Spirulina* sp, were tried out. In addition to that, combinations of *C. calcitrans* + 700 I.U. cod liver oil and *I. galbana* + 700 I.U. cod liver oil were fed to the mussels to test the role of lipids in the maturation process in captivity.

To study the effect of salinity on maturation, the mussels were reared in salinites 15 ppt., 20 ppt., 30 ppt., and 38 ± 1 ppt (control).

In the second treatment to study the effect of pH on induction of maturation in captivity, trials were conducted in pH 7, 8.2 (control) and pH 9.

In both these experiments the mussels were fed with *C. calcitrans*.

For all the three induced maturation experiments the treatments were carried out in ambient room temperature 31 ± 1 ° C

and in an air conditioned room (PLATE II B) with water temperature 23 ± 1 ° C.

The animals were subjected to spawning by thermal stimulation on the 20 th , 30 th , 40 th, and 60 th day after starting the trial. Ten animals from each replicate of each treatment were sacrificed to note the gonadal condition visually on each of the spawning days. At the end of the experiments all remaining animals were sacrificed and their gonadal condition noted visually and tabulated as percentage mean ± standard deviation for each stage.

2. 7. Feeding protocol

The feed was given @ 1 l animal $^{-1}$ day $^{-1}$. (microalgal feed cell concentration 1 x 10 ^{6} ml $^{-1}$). Fifty percent of the feed was given at 8: 00 AM and the remaining at 16: 00 hrs to prevent pseudofaeces formation. In the case of adding oil, the oil was thoroughly mixed with the fresh water and then the feed was added. Salinity and pH adjustments were made according to the quantity of feed added so that feed's salinity and pH do not affect the treatment salinity / pH. Hundred percent water exchange was effected daily. The water quality parameters were also monitored daily. Salinity was adjusted with diluting ambient fresh filtered sea water with dechlorinated tap water and measured using a salino refractometer and cross checked with argentometric titration. The pH was adjusted using 0.1 N NaOH and 0.1 N HCl and measured using pH pen and sub samples cross-checked with a pH meter.

2. 8. Statistical analyses

Data on percentage of spawning in conditioning experiment was analysed through a 3 – way Analysis of Variance (ANOVA) using STATISTICA computer software after arcsine transformation of the percentage spawning data. While the data on percentage of spawning in induced maturation experiments was analysed through a 2 – way Analysis of Variance (ANOVA) using EXCEL computer software after arcsine transformation of the percentages to find out whether there was any significant difference ($p < 0.05$) between the various treatments. If the F value of the treatments were significantly different, then the best treatment was found out through pair wise (Students t – test ($p < 0.05$)) comparison of treatment means using Critical Difference (CD).

3. RESULTS

3. 1. Conditioning of mussels

The broodstock conditioning experiments conducted over a period of 2 weeks indicated that temperature and the type of food had a role in the conditioning and spawning of mussels in captivity. The animals irrespective of the treatment failed to spawn on the fifth and tenth day after conditioning. On the 15th day after conditioning spawning was observed in all the treatment except in *Isochrysis galbana* fed animals at ambient temperature at both the salinities tested. *Chaetoceros calcitrans* fed animals at ambient salinity and conditioned at 23 $^{\circ}$C showed the best spawning percentage of 66.67 ± 5.78 % with both males and females spawning in equal percentage (33.33 ± 5.78) (Fig. 1). Details of the spawning percentage in the various treatments are as follows: 33.33 ± 5.78 % spawned in *I. galbana* fed animals at 32 ppt and 23 ° C, 23.33 ± 5.78 % and none in *C. calcitrans* fed and *I. galbana* fed respectively both at 32 ppt and ambient temperature of 31 ° C (Fig. 1).

In the low salinity experiments the trend was similar though the spawning percentage was lower than the control. The spawning percentage was better in low temperature maintained mussels than at high temperature. While 43.33 ± 5.78 % spawned in *C. calcitrans* fed animals at 23 ° C, 23.33 ± 5.78 % spawned in *I. galbana* fed animals at the same temperature, while the percentage of spawning

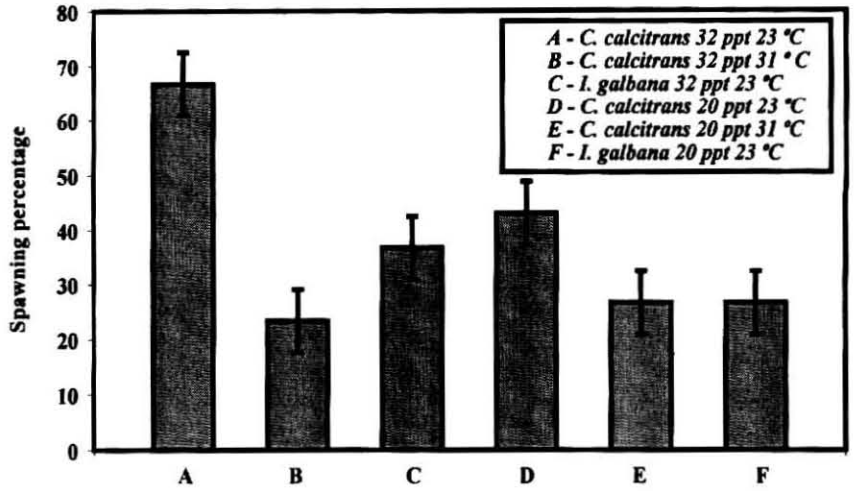


Fig. 1. Percentage spawning of conditioned broodstock of *Perna viridis* at different temperatures, microlagal feed and salinity combinations (vertical bars represents the Standard deviation)

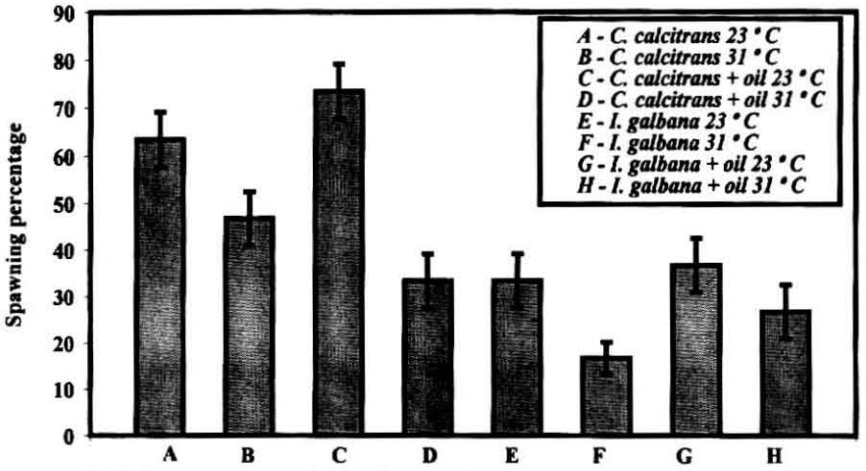


Fig. 2. Percentage spawning in *Perna viridis* broodstock induced to mature in captivity using different feeds (vertical bars represent the standard deviation)

was 26.67 ± 5.77 % and 0 % in animals fed with *C. calcitrans* and *I. galbana* at 31 ° C respectively (Fig.1). Males spawned more in *I. galbana* fed animals than females. No such trend was seen in *C. calcitrans* fed animals.

The reproductive condition of animals at the end of the experimental trial is given in Table 1. *C. calcitrans* fed animals at ambient salinity were found to be in the ripe condition at ambient temperature and spent at low temperature, moreover, *C. calcitrans* fed animals at low salinity showed slow gonadal development to ripeness (Table 1). Even though spawning was achieved (Fig.1) irrespective of the feed and temperature, at low salinity of 20 ppt, it was partial and no healthy viable eggs were produced.

A similar trial conducted at Cochin showed that, animals given the same treatments, spawned without inducement, in all the treatments irrespective of the temperature after 20 days of conditioning. Spawning was observed at night on the new moon day. In this case too, the eggs developed irregularly with the veliger larvae observed after 96 hours and total mortality of the larvae occurring on the 5^{th} day in all the treatments conducted at low salinity.

Statistical tests conducted with spawning data revealed, significant differences between the feeds *C. calcitrans, I. galbana*, the temperatures of 23 ° C and 31 ° C and salinities 32 ppt and 20 ppt. (Table 5). There was significant difference in the percentage spawning between all the treatments. *i.e.* percentage spawning

Table 1. Percentage of reproductive stages of conditioned *Perna viridis* after 15 days (values represent mean ± standard deviation)

Stages	*Chaetoceros calcitrans*				*Isochrysis galbana*			
	32 ppt 24 °C	32 ppt 31 ° C	20 ppt 24 °C	20 ppt 31 °C	32 ppt 24 °C	32 ppt 31 °C	20 ppt 24 °C	20 ppt 31 °C
II	0	0	23.33 ± 5.77	63.33 ± 5.77	53.33 ± 5.77	100	43.33 ± 5.77	100
III	33.33 ± 5.77	76.67 ± 5.77	33.33 ± 5.77	10	10	0	30	0
IV	66.67 ± 5.77	33.33 ± 5.77	43.33 ± 5.77	26.67 ± 5.77	36.67 ± 5.77	0	26.67 ± 5.77	0

between the feeds *C. calcitrans* and *I. galbana* and between the salinity levels 32 and 20 ppt. Significant differences existed between *C. calcitrans* and *I. galbana* at 23 ° C and 31 ° C, also, there was significant difference between the temperatures tested for each of the feed. *C. calcitrans* at 23 ° C at 32 ppt was the best for the conditioning of *Perna viridis* broodstock.

3. 2. Induced maturation

C. calcitrans fortified with cod liver oil at low temperatures of 23 ° C was the best feed for hastening maturity in *Perna viridis* in captivity which resulted in 73.33 ± 5.77 % of the mussels to spawn on the 32nd day, but the same feed combination at 31 ° C was able to spawn only 33.33 ± 5.77 % of the animals on the 37th day. However, non fortified *C. calcitrans* at low temperature was equally good in the induction of maturation of *P. viridis* where 63.33 ± 5.77 % spawned on the 40th day at 23 ° C while 46.67 ± 5.77 % of the animals spawned after thermal stimulation on the 50th day at ambient temperature of 31 ° C (Fig. 2).

Supplemented or non supplemented *I. galbana* was inferior in inducing maturity in *P. viridis* when compared to *C. calcitrans*. Cod liver oil supplemented *I. galbana* fed animals spawned on day 43 (36.67 ± 5.77 %) at 23 ° C whereas 26.67 ± 5.77 % did after 55 days at 31 ° C (Fig. 2). Non supplemented *I. galbana* fed animals spawned only at low temperature on day 48 (33.33 ± 5.77 %) while a poor 16.67 ± 3.85 % spawned at ambient temperature of 31 ° C on day 55. Except for non supplemented *I. galbana* fed animals at

31 ° C, in all the above cases, the spawned gametes were viable. None of the other feeds *viz.*, *Chlorella salina* and *Spirulina* sp., were able to stimulate spawning in *P. viridis* even after 60 days (Table 2). A simultaneous set of animals kept in natural conditions on a raft (wild condition) spawned (86.67 ± 5.77 %) on the 20th day.

It was found that *Isochrysis galbana*, *Chaetoceros calcitrans* fortified and non fortified at low and ambient temperatures and *Chlorella salina* at low temperature was able to mature and ripen *P. viridis* broodstock. The percentages of mussels in the various reproductive stages from stage I to IV have been tabulated for each feed in Table 2.

P. viridis showed a distinct preference for high saline conditions for the induction of maturation and spawning. Mussels maintained in low salinity of 15 ppt under low or ambient temperatures failed to mature or spawn (all were dead by 30 - 40 days). Among the specimens maintained at 20 ppt only 30 % spawned partially at 23 ° C on the 50th day, whereas 53.33 ± 5.77 % matured and spawned at 30 ppt on the 46th day. 63.33 ± 5.77 % spawned at 23 ° C in the control on the 40th day. At ambient temperature the percentage of spawning was none in 20 ppt, 43.33 ± 5. 77 % at 30 ppt, 46.67 ± 5.77 % in control salinity on the 53rd and 50th days respectively (Fig. 3).

The percentages of the various reproductive stages from stage I to IV have been tabulated for each salinity in Table 3.

pH also was a limiting factor at very high and low ranges (9, 6) while the results were nearly the same at ambient range of 7 – 8 in the inducement of maturation and spawning in *P. viridis* . At pH 7, animals maintained at low temperature of 23 ° C spawned (56.67 ± 5.77 %) on the 46th day while in controls 63.33 ± 5.77 % spawned on the 40th day (Fig. 4). All the animals exposed to pH 9 died between days 30 and 40. At ambient temperature of 31 ° C, the percentage of spawning ranged from 33.33 ± 5.77 % and 46.67 ± 5.77 % for pH 7 and control pH of 8.2 on the 53rd and 50th days respectively (Fig. 4).

The percentages of the various reproductive stages from I to IV have been tabulated for each pH in Table 4.

Statistical tests showed that induction of maturation was significantly different for the different microalgae tested (Table 6). *C. calcitrans* either fortified with or without cod liver oil at 23 ° C was the best for the maturation induction in *Perna viridis*.

Induction of maturation was significantly different for the different salinities and temperatures tested (Table 7). Control salinity of 38 ppt at 23 ° C was the best for the induction of maturation in *P. viridis*.

Induction of maturation was not significantly different between the different pH tested, but was significantly different for the two temperatures used (Table 8). pH 7 - 8.2 at 23 ° C was the best for the maturation induction in *P. viridis*.

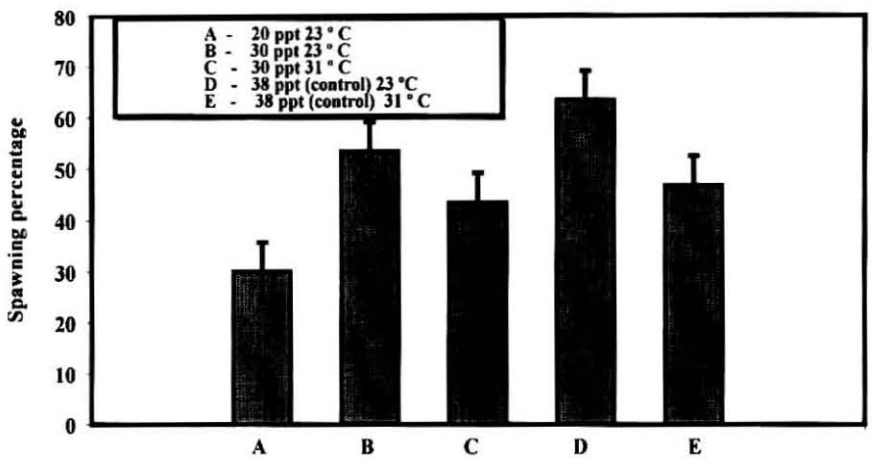


Fig. 3. Percentage spawning in *Perna viridis* broodstock induced to mature in captivity using different salinity (vertical bars represent standard deviation)

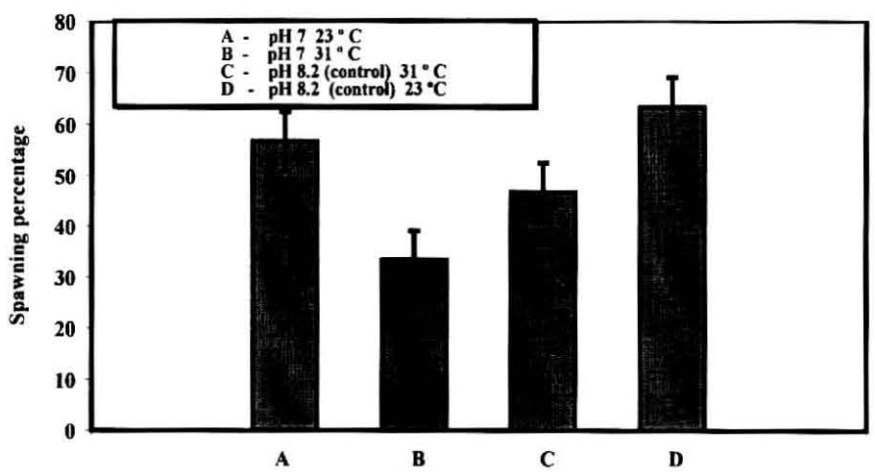


Fig. 4. Percentage spawning in *Perna viridis* broodstock induced to mature in captivity using different pH (vertical bars represent the standard deviation)

Table 2. Effect of different feeds on the induced maturation in *Perna viridis*
Percentage of reproductive stages on days 20,30,40 and 60
I : Early Maturing; II: Mature; III: Ripe; IV: Spent

Day	Feed	23°C				31°C			
		Reproductive Stages				Reproductive Stages			
		Mean ± S.D				Mean ± S.D			
		I	II	III	IV	I	II	III	IV
20	*Chaetoceros calcitrans*	56.67 ± 5.77	43.33 ± 5.77	0	0	86.67 ± 5.77	13.33 ± 5.77	0	0
	C.calcitrans + oil	46.67 ± 5.77	33.33 ± 5.77	20	0	76.67 ± 5.77	23.33 ± 5.77	0	0
	Isochrysis galbana	46.67 ± 5.77	53.33 ± 5.77	0	0	86.67 ± 5.77	13.33 ± 5.77	0	0
	I. galbana + oil	43.33 ± 5.77	56.67 ± 5.77	0	0	86.67 ± 5.77	13.33 ± 5.77	0	0
	Spirulina sp.	86.67 ± 5.77	13.33 ± 2.46	0	0	100	0	0	0
	Chlorella salina	90	10	0	0	90	10	0	0
	No Feed	100	0	0	0	100	0	0	0
30	*Chaetoceros calcitrans*	13.33 ± 5.77	30 ± 5.77	56.67 ± 5.77	0	66.67 ± 5.77	33.33 ± 5.77	0	0
	C.calcitrans + oil	16.67 ± 5.77	26.67 ± 5.77	56.67 ± 5.77	0	33.33 ± 5.77	46.67 ± 5.77	20	0
	Isochrysis galbana	33.33 ± 5.77	66.67 ± 5.77	0	0	76.67 ± 5.77	23.33 ± 5.77	0	0
	I. galbana + oil	20	70	10	0	36.67 ± 5.77	63.33 ± 5.77	0	0
	Spirulina sp.	66.67 ± 5.77	33.33 ± 5.77	0	0	90	10	0	0
	Chlorella salina	83.33 ± 5.77	16.67 ± 5.77	0	0	90	10	0	0
	No Feed	90	10	0	0	100	0	0	0
40	*Chaetoceros calcitrans*	0	0	36.67 ± 5.77	63.33 ± 5.77	0	53.33 ± 5.77	46.67 ± 5.77	0
	C.calcitrans + oil	0	0	26.67 ± 5.77	73.33 ± 5.77	0	26.67 ± 5.77	40	33.33 ± 5.77
	Isochrysis galbana	0	53.33 ± 5.77	46.67 ± 5.77	0	10	60	30	0
	I. galbana + oil	0	46.67 ± 5.77	53.33 ± 5.77	0	0	76.67 ± 5.77	23.33 ± 5.77	0
	Spirulina sp.	46.67 ± 5.77	53.33 ± 5.77	0	0	56.67 ± 5.77	43.33 ± 5.77	0	0
	Chlorella salina	43.33 ± 5.77	56.67 ± 5.77	0	0	56.67 ± 5.77	43.33 ± 5.77	0	0
	No Feed	86.67 ± 5.77	13.33 ± 5.77	0	0		0	0	0
60	*Chaetoceros calcitrans*	0	0	0	0	10	10	33.33 ± 5.77	46.67 ± 5.77
	Isochrysis galbana	0	23.33 ± 5.77	43.33 ±5.77	33.33 ± 5.77	0	36.67 ± 5.77	46.67 ± 5.77	16.67 ± 3.47
	I. galbana + oil	0	26.67 ± 5.77	36.67 ± 5.77	36.67 ± 5.77	0	20	53.33 ± 5.77	26.67 ± 5.77
	Spirulina sp.	23.33 ± 5.77	76.67 ± 5.77	0	0	53.33 ± 5.77	46.67 ± 5.77	0	0
	Chlorella salina	33.33 ± 5.77	50	16.67 ± 3.46	0	33.33 ± 5.77	66.67 ± 5.77	0	0

Table 3. Effect of salinity on the induced maturation in *Perna viridis*
Percentage of reproductive stages on days 20, 30, 40 and 60
I : Early Maturing; II: Mature; III: Ripe; IV: Spent

Day	Salinity (ppt)	23°C				31°C			
		Reproductive Stages				Reproductive Stages			
		Mean ± S.D				Mean ± S.D			
		I	II	III	IV	I	II	III	IV
20	15	100	0	0	0	100	0	0	0
	20	56.67 ± 5.77	0	0	0	100	0	0	0
	30	43.33 ± 5.77	56.67 ± 5.77	0	0	63.33 ± 5.77	36.67 ± 5.77	0	0
	38 (Control)	56.67 ± 5.77	43.33 ± 5.77	0	0	86.67 ± 5.77	13.33 ± 5.77	0	0
30	15	90	10	0	0	100	0	0	0
	20	43.33 ± 5.77	56.67 ± 5.77	0	0	73.33 ± 5.77	26.67 ± 5.77	0	0
	30	23.33 ± 5.77	66.67 ± 5.77	10	0	23.33 ± 5.77	76.67 ± 5.77	0	0
	38 (Control)	13.33 ± 2.46	30	56.67 ± 5.77	0	66.67 ± 5.77	33.33 ± 5.77	0	0
40	20	20	53.33 ± 5.77	26.67 ± 5.77	0	46.67 ± 5.77	53.33 ± 5.77	0	0
	30	20	23.33 ± 5.77	56.67 ± 5.77	0	0	43.33 ± 5.77	56.67 ± 5.77	0
	38 (Control)	0	0	36.67 ± 5.77	63.33 ± 5.77	0	53.33 ± 5.77	46.67 ± 5.77	0
60	20	0	20	46.67 ± 5.77	30	10	50	40	0
	30	0	10	36.67 ± 5.77	53.33 ± 5.77	0	23.33 ± 5.77	33.33 ± 5.77	43.33 ± 5.77
	38 (Control)					10	10	33.33 ± 5.77	46.67 ± 5.77)

Table 4. Effect of pH on the induced maturation in *Perna viridis*
Percentage of reproductive stages on days 20, 30, 40 and 60
I : Early Maturing; II: Mature; III: Ripe; IV : Spent

Day	pH	23° C				31° C			
		Reproductive Stages				Reproductive Stages			
		Mean ± S.D				Mean ± S.D			
		I	II	III	IV	I	II	III	IV
20	7	53.33 ± 5.77	46.67 ± 5.77	0	0	80	20	0	0
	8.2 (Control)	56.67 ± 5.77	43.33 ± 5.77	0	0	86.67 ± 5.77	13.33 ± 5.77	0	0
	9	86.67 ± 5.77	13.33 ± 5.77	0	0	100	0	0	0
30	7	33.33 ± 5.77	66.67	0	0	43.33 ± 5.77	56.67 ± 5.77	0	0
	8.2	13.33 ± 5.77	30	56.67 ± 5.77	0	66.67 ± 5.77	33.33 ± 5.77	0	0
	9	73.33 ± 5.77	26.67 ± 5.77	0	0	100	0	0	0
40	7	0	43.33 ± 5.77	56.67 ± 5.77	0	20	43.33 ± 5.77	36.67 ± 5.77	0
	8.2	0	0	36.67 ± 5.77	63.33 ± 5.77	0	53.33 ± 5.77	46.67 ± 5.77	0
60	7	0	0	36.67 ± 5.77	63.33 ± 5.77	0	43.33 ± 5.77	23.33 ± 5.77	33.33 ± 5.77
	8.2					10	10	33.33 ± 5.77	46.67 ± 5.77

Table 5. Conditioning of broodstock of *Perna viridis* at different temperatures, microlagal feed and salinity combinations

3- way Analysis of Variance (3 - way ANOVA)

		SS	df	MS	F	Sig.
Main Effects	FEED	2727.47	1.00	2727.47	130.02	0.00 *
	SAL	239.72	1.00	239.72	11.43	0.00 *
	TEMP	4260.54	1.00	4260.54	203.11	0.00 *
2-Way Interactions	FEED * SAL	20.55	1.00	20.55	0.98	0.34
	FEED * TEMP	223.69	1.00	223.69	10.66	0.00 *
	SAL * TEMP	147.86	1.00	147.86	7.05	0.02 *
Residual		356.61	17.00	20.98		

Table 6. Effect of feeds on the induced maturation of *Perna viridis*

2- way Analysis of Variance 2 - way ANOVA)

Source of Variation	SS	df	MS	F	F crit	P-value
Feeds	4543.95	3.00	1514.65	56.89	3.24	0.00 *
Temperature	4996.53	1.00	4996.53	187.67	4.49	0.00 *
Interaction	459.09	3.00	153.03	5.75	3.24	0.01 *
Within	425.98	16.00	26.62			

Table 7. Effect of salinity on the induced maturation of *Perna viridis*

2- way Analysis of Variance (2 - way ANOVA)

Source of Variation	SS	df	MS	F	F crit	P-value
Salinity	3362.48	2.00	1681.24	110.50	3.89	0.00 *
Temperature	1956.88	1.00	1956.88	128.61	4.75	0.00 *
Interaction	423.74	2.00	211.87	13.92	3.89	0.00 *
Within	182.58	12.00	15.22			

Table 8. Effect of pH on the induced maturation of *Perna viridis*

2- way Analysis of Variance (2 - way ANOVA)

Source of Variation	SS	df	MS	F	F crit	P-value
pH	22.22	1.00	22.22	0.34	5.32	0.58
Temperature	1200.20	1.00	1200.20	18.16	5.32	0.00 *
Interaction	42.98	1.00	42.98	0.65	5.32	0.44
Within	528.77	8.00	66.10			

*** significant at $p < 0.05$**

Thus *Perna viridis* broodstock fed with *Chaetoceros calcitrans* at 23 ° C at 32 ppt can be conditioned to mature in 15 days. Inducement of maturation was also achieved by the 32nd day by feeding immature or early maturing *P. viridis* with *C. calcitrans* fortified with 700 I.U. cod liver oil at 23 ° C at high salinity above 30 ppt and pH 7 – 8.2.

4. DISCUSSION

In marine bivalves, gametogenesis can be induced outside the normal reproductive period and in animals already undergoing gametogenesis, gamete development can be accelerated. Loosanoff and co - workers demonstrated that elevated temperature stimulated the development of gonads of certain commercial molluscs (Loosanoff and Davis, 1950, 1952, 1963). In mussels, this technique was first demonstrated by Bayne (1965) in *Mytilus edulis*.

In *P. viridis,* maturity was achieved in specimens conditioned at low temperature of 23 ° C with an adequate diet of *Chaetoceros calcitrans* at ambient high salinity of 32 ppt and none at 32 ppt and 31 ° C. The results are in contrast to the results of AQUACOP (1979) and Siddall (1980), Sreenivasan *et al.* (1988 *a*) who conditioned mussels *M. viridis* and *P. viridis* to spawn at ambient temperatures of 28 - 30 ° C. Chotipuntu and Pongthana (2000) also reported the same for *Crassostrea belcheri.* On the other hand, conditioning of broodstock at low temperature to mature has been successful in *Crassostrea madrasensis* (Nayar *et al.*, 1984, 1987, 1988; Palaniswamy and Sathakathullah, 1992) and *Pinctada fucata* (Alagarswami *et al.*, 1987) where the authors have used a mixed algal feed predominated by *Chaetoceros* sp.

In temperate species of mussels, Bayne (1965), Hrs – Brenko (1973 *b*) used *Phaeodactylum tricornutum*, while Sprung (1984 *a – d*) used a mixture of *Dunaliella tertiolecta* and *Isochrysis galbana* for

conditioning *M. edulis*. Recent works have also stressed the role of conditioning of bivalves for successful spawning using different microalgae like *Isochrysis galbana, Pseudolsochrysis* sp, T- *Isochrysis, Pavlova lutheri* (DiSalvo *et al.*, 1983; Wilson *et al.*, 1996; Numaguchi, 1997; Utting and Millican, 1997, 1998; Buchannan *et al.*, 1998; Kent *et al.*, 1998; Trotia and Cordisco, 1998; Jeffs, 1999).

Results of experiments conducted to induce maturation in *P. viridis* indicated clearly that *Chaetoceros calcitrans* (fortified with cod liver oil and non-fortified) fed to animals maintained at low temperature gave the best result. Effect of low temperature on hastening maturity in *P. viridis* is in agreement with the works of Velez and Epifano (1978) in *Perna perna* and Coeroli *et al.* (1984), Jeffs (1999) for other bivalve species. On the other hand, Ajithakumar (1984) obtained maturation of *Perna indica* at ambient temperature 28 – 30 ° C and Bayne *et al.* (1982) found a mixed diet of *Phaeodactylum* and *Tetraselmis* best suitable for the maturation of *M. edulis*.

Chaetoceros calcitrans as a feed has been found to be the best food for the maturation of *P. viridis* when compared to other microlagae like *I. galbana, Spirulina* sp. and *Chlorella salina*. Success in conditioning and maturation of mussels and other bivalves is quantified in the quality of the eggs and larvae produced (Gallager and Mann, 1986; Gallager *et al.*, 1986). Larval growth, survival, settlement spat and juvenile growth in mussels are influenced by egg quality (Bayne, 1972, Bayne *et al.*, 1975, 1982).

Bayne *et al.* (1975) had reported that lipid content of the eggs is dependent on the food ration available to the *female during* gametogenesis. The importance of lipid in the maturation process of bivalve broodstock has been emphasized in detail by Utting (1993) and Utting and Millican (1997) who were of the opinion that best diets for conditioning and maturation of broodstock are those which contain high amounts of Poly Unsaturated Fatty acids (PUFA), Eicosapentaenoic acid (EPA) and Docosahexaenoic acid (DPA) . Since adults of bivalves of most species are unable to produce these *de novo* from short chain fatty acids, these must be provided in the diets.

EPA and DHA are essential for the embryonic development and structural functions respectively. The quantity and quality of the gametes is reflected in the broodstock conditioning especially in the lipid content (Bayne, 1972; Bayne *et al.*, 1975). *Chaetoceros* is rich in essential fatty acids mainly of EPA whereas T – *Isochrysis galbana* is rich in DHA (Volkman *et al.*, 1989; Brown *et al.*, 1997). In *P. viridis*, both in conditioning and maturation experiments, the earlier maturation and spawning obtained in *C. calcitrans* fed animals especially in those animals fed with fortified cod liver oil may be due to this reason. Thus the better results obtained in non fortified *C. calcitrans* fed mussels than fortified *I. galbana* for *P. viridis* maturation show clearly that *Chaetoceros calcitrans* is the best feed for the conditioning and maturation of *P. viridis* in captivity.

Again in *P. viridis*, both in the conditioning and maturation studies, the mussels spawned spontaneously in high salinity. This is

in agreement with the work of Bayne *et al.* (1978) in *M. edulis*, Muranaka and Lannan (1984) for *Crassostrea gigas* and Wilson (1968, 1969) for *Xenostrobus securis*. Maturation in high salinity has also been reported for *P. viridis* in the wild (Sreenivasan *et al.*, 1989 *b*; Rajagopal *et al.*, 1997, 1998 *a, b*).

CHAPTER II

INDUCED SPAWNING OF *PERNA VIRIDIS*

1. INTRODUCTION

Controlled spawning of bivalves has received considerable attention in recent years mainly due to the need for augmenting the timely and steady supply of quality seed for commercial shellfish farming and as well as sea ranching operations to enhance natural population. Controlled breeding of bivalves is of strategic importance to India in the present context of enhanced activities in the culture of commercially important shellfishes such as edible oysters, mussels, pearl oysters and clams.

There are exhaustive reviews on the various methods employed for the inducement of spawning in bivalve molluscs (Loosanoff and Davis, 1963; Ino, 1972; Giese and Pearse, 1974; Sastry, 1979; Alagarswami, 1980; LePennec, 1981; Lutz and Kennish, 1992; Snodden and Roberts, 1997). Very often spawning occurs under different conditions such as the change of environment, mere mechanical handling of the molluscs, immersion in sea water after transportation and also changes in water temperature, salinity or pH in *M. edulis* and *Perna viridis* (Just, 1939; Berg and Kutsky, 1951; Sreenivasan *et al.*, 1988 *a*).

Several workers have tried out different mechanical methods like, shaking violently, touching or pricking of the adductor muscle,

pegging, shell scrapping, removing one or both shell valves to spawn mussels *M. californianus, M. edulis, M. galloprovincialis* and *M. viridis* (Young, 1945; Loosanoff and Davis, 1963; Hrs-Brenko and Calabrese, 1969; Hrs-Brenko, 1973 *b*, 1974 *a*; Masson, 1975; Tan, 1975 *a*; Rao *et al.*, 1976).

Desiccation alone or in combination with thermal stimulation has been tried out with success to spawn mussels like *M. edulis, M. galloprovincialis, M. chilensis* and *P. indica* (Berg and Kutsky, 1951; Bayne, 1965; Dixon, 1982; Roy and Joseph, 1992; Scarpa *et al.*, 1994 *b*; Toro and Sastre, 1995; Kiyomoto *et al.*, 1996).

Thermal stimulation or shock by sudden increase or decrease of ambient temperature has been the commonest method adopted in spawning several species of molluscs, particularly those in temperate and subtropical regions. Loosanoff and Davis (1963) used this method to spawn a large number of molluscs. Several workers have employed this method to spawn mussels like *M. edulis* (Iwata 1951 *d*; Sprung, 1984 *a* - *d*; Sprung and Bayne, 1984; Eyster and Pechenik, 1987; Beaumont and Kelly, 1989; Pechenik *et al.*, 1990; Medakovic, 2000), *M. edulis aoteanus* (Tortell, 1980; Redfearn *et al.*, 1986), *M. galloprovincialis* (Masson, 1975; His *et al.*, 1989; Scarpa *et al.*, 1993, 1994 *a*; Satuito *et al.*, 1994, 1995; Komaru *et al.*, 1995; Kiyomoto *et al.*, 1996), *M. edulis galloprovincialis* (Satuito *et al.*, 1997, 1999), *M .viridis* (Tan, 1975 *a* ; Rao *et al.*, 1976; Sivalingam, 1977; AQUACOP, 1979, 1983), *P. perna* (Siddall, 1979 *a, b*, 1980, 1982; Velez and Azuaje, 1993), *P. canaliculus*

(Tortell, 1980; Redfearn *et al.*, 1986), *P. viridis* (Rengarajan, 1983 *a*; Sreenivasan *et al.*, 1988 *b*), *P. indica* (Appukutan *et al.*, 1984, 1988) and in *Modiolus modiolus* (Lutz and Hidu, 1979) .

Experiments on spawning with salinity shock or by salinity variations have been tried by an increase or decrease of salinity in mussels *M. edulis, M. viridis, P. viridis, P. indica* and *Dreissena polymorpha* (Loosanoff and Davis, 1963; Sivalingam, 1977; Stephen and Shetty, 1981; Fong *et al.*, 1995) .

Several workers have reported spawning at alkaline pH in mussels (Sagara, 1958; Iwata, 1951 *a* - *c*; Morse *et al.*, 1977 *b*, 1978; Sivalingam, 1977; Breese and Robinson, 1981; Smith and Strehlow, 1983; Trevelyan and Chang, 1983).

Hydrogen Peroxide has been reported to be effective in stimulating release of gametes singly or in combination with other stimuli in *M. edulis* and *M. californianus* (Morse *et al.*, 1977 *b*, 1978; Trevelyan and Chang, 1983).

Morse *et al.* (1977 *b*) has found addition of Tris (Tris-hydroxymethylamine methane) buffer to make alkaline sea water pH 9.1 alone induced *M. edulis* and *M. californianus* to spawn.

Injection of millimolar solution of Potassium Chloride (KCl) into the posterior adductor muscle or the mantle cavity of the mussels have been tried out *M. edulis, M. viridis* (Iwata, 1951 *a* - *c*;

Bayne, 1965; Bayne *et al.*, 1975, 1978; Rao *et al.*, 1976; Beaumont and Budd, 1982; Yamamoto and Sugawara, 1988; Beaumont and Kelly, 1989).

Serotonin in low doses when added to sea water or injected was found to induce spawning in *P. viridis* and *Dreissena polymorpha* (Ram *et al.*, 1993; Fong *et al.*, 1996; Tan, 1997; Fong, 1998).

It has been well established that adding eggs or sperm suspension can induce spawning in many bivalves. In mussels too it has been used with mixed results (Miyazaki, 1935; Young, 1945; Chipperfield, 1953; Lubet, 1959; Loosanoff and Davis, 1963; Bayne, 1965; Culliney, 1971; Rao *et al.*, 1976; Schweinitz and Lutz, 1976; Sivalingam, 1977; Stephen and Shetty, 1981; His *et al.*, 1989; Roy and Joseph, 1992).

In this study, induced spawning was tried out in *Perna viridis*. The experiments carried out included the effect of different salinity, pH, desiccation, combined effect of temperature and salinity and pH. The effect of different millimolar chemicals like Tris, Hydrogen peroxide, Serotonin and Potassium chloride was tested. The effect of sperms or egg suspension alone or in combination with temperature stimulation was also tried.

2. MATERIAL AND METHODS

2.1. Experimental protocol

For studies on natural spawning of *Perna viridis* specimens of 70 ± 2 mm in shell length were used. All the experiments were conducted in the Shellfish hatchery at Tuticorin Research Centre of C.M.F.R.I., Tuticorin in August 1999. For this experiment mussel ropes were taken from a culture rack put in the sea near the shellfish hatchery. Individual mussels were carefully cut from the cultured rope in such a way as to prevent damage to the byssus threads. Thirty mussels attached to a rope were used as the control. Individually detached mussels were then graded and cleaned and used for the various experiments

In all the spawning experiments thirty ripe mussels (10 x 3) in triplicate, were put into 15 l tubs containing 10 l sea water and the number of animals spawned were noted. The experiment was stopped after 24 hours. The results were tabulated as percentage mean ± S.D. for spawned males and females together and separately. The results were statistically treated. Here, the criteria for positive spawning (PLATE IV B) was the release of gametes for continuous period of half an hour and any spawning less than 15 minutes was considered as partial spawning. Once spawned, the gametes were removed into 100 ml beakers containing ambient sea water to test the viability of the gametes. The viability of the spawning was tested by the activity of the gametes for one hour. If

PLATE IV

A. Spawning male *Perna viridis*
(Arrow indicates release of sperms)

B. Spawning female *Perna viridis*

fertilized, the formation of morula stage (PLATE V) was taken as a positive spawning response and if unfertilized then the sperms were checked for motility and in eggs, for their shape.

2.2. Desiccation

Cleaned mussels from the farm were kept in the shade out of water (air temperature 32 ° C for one hour and then immediately transferred to ambient temperature sea water taken from the same source and observed for spawning.

2.3. Natural spawning at different salinities.

To determine the threshold salinity for spawning in mussels, salinity regimes of 15, 20, 25, 30, 38 (Control) and 45 ppt. were prepared. The lower sea water dilutions were prepared by diluting ambient sea water with dechlorinized tap water while, the higher salinity of 45 ppt was prepared by adding sterilized brine to ambient sea water. The salinities prepared were checked with a salinity refractometer and counter checked by argentometric titration (Strickland and Parsons, 1972) and adjusted accordingly before introducing the mussels.

2. 4. Natural spawning in different pH regimes

Cleaned mussels were subjected to different pH regimes prepared by adding necessary quantities of 0.1 N HCl to get pH values of 7 and 6 and using 0.1 N NaOH to get pH value of 9. The ambient pH of 8.2 was used as the control.

2.5. Combination of salinity variations and thermal manipulation

To find out the combined effects of salinity and temperature on spawning, different salinities were made as that mentioned before in the natural spawning experiments. The temperature was raised by 5 ° C from the ambient (31 ° C to 36 ° C) in each of these tubs by adding hot sea water of the respective salinity. The experiment was stopped at the end of 24 hours and the number of mussels spawned was noted.

2.6. Combination of pH variations and thermal manipulation

In this experiment the protocol employed was the same as that of salinity variations. Ambient seawater of pH 8.2 was adjusted to prepare sea water of pH 6, 7 and 9 and decreased using previously mentioned protocol on pH manipulation. The mussels were then introduced and observed for spawning.

2.7. Chemicals

2.7.1. Hydrogen Peroxide (H_2O_2)

Hydrogen peroxide (Qualigens, Glaxo) 30 % w/v stored at 4 ° C was diluted to 6 % w/v stock solution with distilled water. Using this stock solution , test concentrations of 5 mM, 6 mM, 10 mM and 30 mM each were prepared in 30 l fresh filtered sea water. Controls were maintained in ambient sea water without any chemical addition.

After 3 hours the animals were transferred to tubs containing fresh filtered sea water. The experiment was terminated after 24 hours.

2.7.2. Tris - Hydroxymethyl Aminomethane (Tris)

Sea water of ambient pH and salinity (8.2 ; 38 %.) was made alkaline by adding Tris. Crystals of Tris (Sisco Research Ltd.) were dissolved in distilled water and added to sea water slowly by means of a dropper and constant stirring to make 30 l each of the test pH solutions of pH 8.5 , 8.8, 9, 9.1, 9.5. The rest of the protocol was same as that conducted for the H_2O_2 experiment.

2.7.3. Combination of Tris and H_2O_2

To find out the combined effect of Tris and H_2O_2, concentrations of 2 mM, 5 mM, 10 mM, 20 mM and 30 mM H_2O_2 were prepared in alkaline sea water of pH 9.1 using Tris. A control was also maintained. The rest of the protocol was same as that conducted for the H_2O_2 experiment.

2.7.4. Serotonin

Serotonin (5 - Hydroxy Tryptamine , Sigma) stock solution of 1 Molar was prepared in fresh filtered sea water . From this stock solution a 2mM solution was prepared. 0.5 ml of this solution was 1) injected into the posterior adductor muscle using a sterilized hypodermic syringe and a 20 G needle and 2) added to the sea water medium to have a final concentration of 2 mM and 5 mM into which mussels were added. Controls were injected 0.5 ml of filtered

sea water and others maintained in ambient sea water conditions. The rest of the protocol was same as that conducted for the previous experiments.

2.7. 5. Potassium Chloride (KCl)

Granules of KCl (Qualigens, Glaxo) were dissolved in distilled water to make a stock solution of 1 M concentration. From this stock solution millimolar solutions of 2 and 5 mM were prepared in fresh filtered sea water. The rest of procedure was similar to the one done for Serotonin experiment.

2.8. Biological Stimulation

2.8.1. Preparation of sperm and egg suspensions

For this a ripe mussel was sacrificed and 1g of the mantle was cut using a sterilized scissors and finely homogenized with a pestle and mortar adding a little sterilized sea water. The solution was then transferred to a sterilized 20 ml test tube and added 3 ml fresh filtered sea water and allowed to stand. The supernatant was pipetted out into another test tube. One ml of this solution was taken into a haemocytometer and the sperm count taken. The sperm count was adjusted to 1×10^{6} sperms ml^{-1}. Two millilitres of this suspension was added to each of the tubs and the animals observed for spawning. A similar methodology was employed for preparation of egg suspension too.

2.8.2. Combination of gamete suspension and thermal manipulation

A protocol similar to the earlier one was adopted here except that after addition of sperm / egg suspension, a thermal shock of 5 ° C was also administered. The number of animals used and other protocols were similar to earlier mentioned protocols for thermal stimulation and gamete addition.

2.9. Statistical analyses

Data on percentage of spawning was analysed through a 2 – way Analysis of Variance (ANOVA) with SPSS 4 computer software after arcsine transformation of the percentages for the three categories separately and also between the main treatments and categories (only for natural and temperature stimulated treatments). 1. Natural spawning, 2. Temperature stimulated spawning and 3. Chemical stimulated spawning. In all the cases, when the F value of the treatments were significantly different, the best treatment was found out through pair wise (Students t – test ($p < 0.05$)) comparison of treatment means using Critical Difference (CD).

3. RESULTS

3.1. Natural spawning

3.1.1. Effect of desiccation on natural spawning

Desiccation did not improve the spawning percentage when compared to the control. Spawning observed was to the tune of 70 ± 7.07 % in desiccated mussels. Females responded better than males (67.13 ± 4.91 % for males and 91.67 ± 7.07 % for females). In the control, 66.67 ± 5.77 spawned (Fig. 5).

Statistical treatment showed that there was no significant difference between the percentage of spawning among the two sexes but the spawning percentage was significant among the various treatments as observed one each for natural spawning, thermally stimulated spawning and chemically induced spawning (Tables 9, 10, 11). Desiccation did not significantly improve the percentage of spawning in *Perna viridis* when compared to the control.

3.1.2. Natural spawning in different salinities.

Better spawning was observed in higher salinities with highest in the control or ambient salinity of 38 ppt. The percentage of spawning ranged from 16.67 ± 3.45 %, 26.67 ± 2.88 %, 53.33 ± 7.071 %, 60 ± 7.071 % at salinities 15, 20, 25, 30 ppt respectively to 66.67 ± 5.77 % in the control and 53.33 ± 2.89 % at 45 ppt. Not much variation was observed in the percentage of spawning at higher salinites from 25 - 45 ppt. (Fig. 6). Time taken for spawning showed that 1 – 1 ½ hours was

necessary for mussels to initiate spawning at salinities (25 - 38 ppt), while it took 4 - 6 hours for a partial spawning at 15, 20 and 45 ppt.

Spawning was significantly lower at very low salinity of 15 and 20 ppt and very high salinity (45 ppt) when compared to the control (Table 9). The percentage of mussels spawning iln salinities ranging from 25 - 38 ppt was not significantly different.

3.1.3. Natural spawning in different pH range

The results of spawning in varied pH range indicated clearly that mussels need an optimum alkaline medium for spawning. Spawning was significantly lower in acidic pH and very high alkaline pH when compared with the control pH. The combined percentage of spawning ranged from 10 ± 5.77 %, 50 ± 10 %, 53.33 ± 7.071 %, 60 ± 3.53 % and 33.33 ± 3.53 % at pH 6, 7, 8.2 (control), 9.1 and 9.5 respectively (Fig. 7).

pH 6 and pH 9.5 showed significantly very low percentage of spawning when compared to the control and with other pH ranges (Table 9). Percentage of mussels spawned in alkaline to mid alkaline pH did not significantly differ from the control but differed significantly in lower pH ranges. Alkaline to mid alkaline pH (7 – 9.1) was found optimum for the spawning of *P. viridis*.

3.2. Thermal stimulation

3.2.1. Effect of temperature and salinity on spawning of mussels

Results of the combined effects of different salinities and temperature shock on the induced spawning of *P. viridis* revealed a similar pattern as that of natural spawning in different salinities. No sex wise difference was observed in the influence of spawning in *Perna viridis*. Spawning was reduced at lower salinities of 15 and 20 ppt in spite of the temperature shock that was further increased to a maximum at 38 ppt (control) salinity and reduced at a very high salinity of 45 ppt (Fig. 8). Spawning started in 30 - 45 minutes in salinites above 20 ppt with profuse spawning whereas only a partial spawning after 4 and 5 hours was observed in 15, 20 and 45 ppt respectively. Spawning percentage in salinities from 25 to 38 ppt varied little. The percentage of spawning was 40 ± 5.77 %, 50.46 ± 5.77, 86.67 ± 5.77 %, 90 ± 7.07 %, 90 ± 5.77 % and 63.33 ± 7.07 %, for salinities 15, 20,25, 30, 38 (control) and 45 ppt respectively (Fig. 8).

Percentage spawning in the control was significantly better than ones at low salinities of 15, 20 ppt and the highest salinity of 45 ppt (Table 10). The combined effect of temperature and salinity was not significant with the control at higher salinity ranges of 25 - 38 ppt. Percentage of spawning in 25 – 38 ppt did not differ significantly. Thus a salinity range between 25 and 38 ppt with a thermal shock gave the best results.

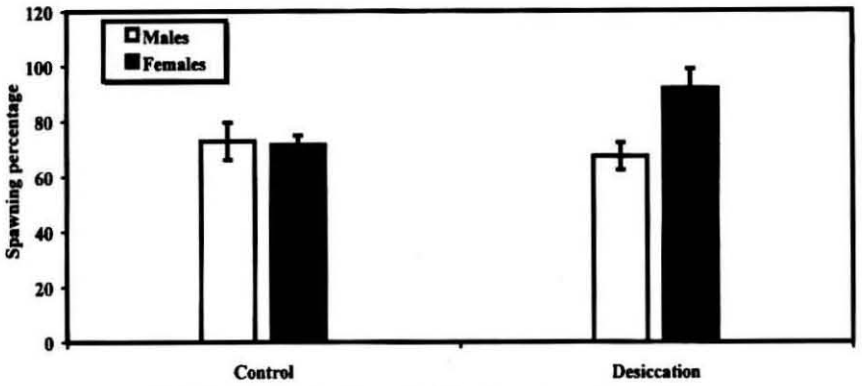


Fig. 5. Effect of desiccation on the induced spawning in *Perna viridis*. (Vertical bars represent standard deviation)

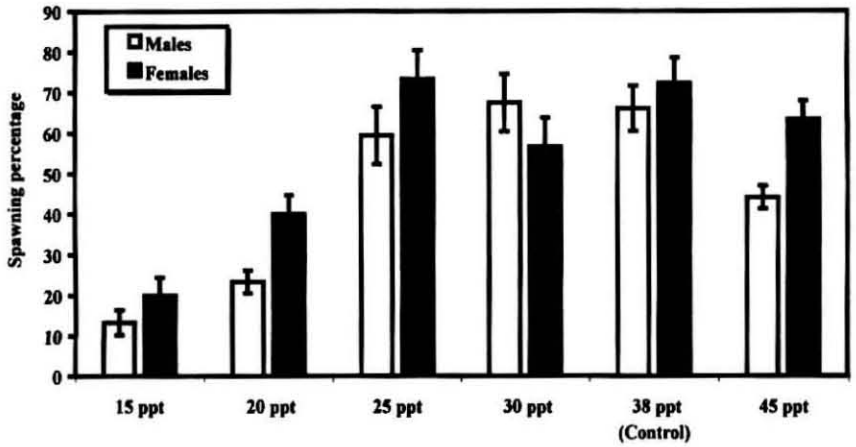


Fig. 6. Effect of salinity variations on the induced spawning in *Perna viridis* (Vertical bars represent standard deviation)

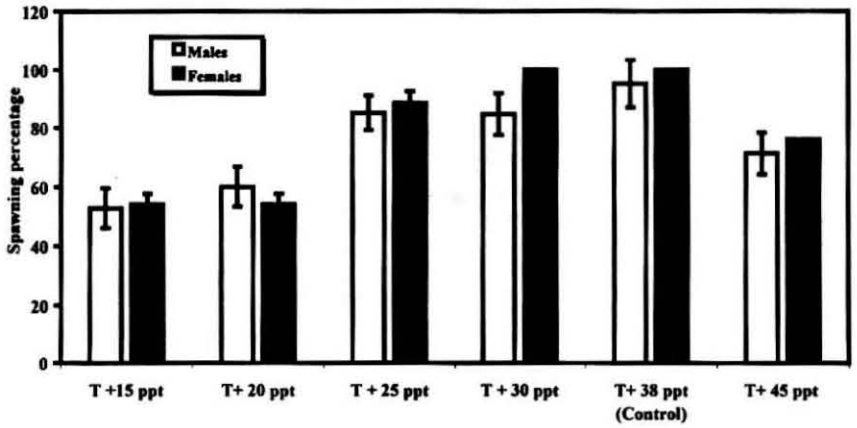


Fig. 7. Combined effect of temperature and salinity on the induced spawning in *Perna viridis*.(Vertical bars represent standard deviation)

3.2.2. Combined effect of temperature and pH

From neutral pH to high alkaline pH the combination with temperature stimulation resulted in a marked increase of spawning. Extreme pH values of pH 6 and pH 9.5 even in combination with a temperature shock failed to elicit a full spawning response in mussels. All mussels in alkaline range (except pH 9.5) spawned profusely within 2 hours while partial spawning was observed in pH 6 and very high pH of 9.5 that too after 18 hours. As in the case of natural spawning at different pH ranges, best results were observed in pH 9.1. The percentage of spawning ranged from 26.67 ± 5.77 %, 60 ± 7.07 %, 63.33 ± 7.071 %, 73.33 ± 7.071 % and 46.67 ± 5.77 % for pH 6, 7, 8.2 (control), 9.1 and 9.5 respectively (Fig. 9).

Percentage spawning at different pH was similar to the experiments on natural spawning in different pH ranges. The percentage spawning in controls was significantly better than the treatment combination of temperature stimulation and low pH of 6 and very high alkaline pH of 9.5 (Table 10). The spawning percentage in pH 9.5 was significantly lower than that of other pH ranges of 7, 8.2 and 9.1. There was no significant difference in the percentage spawning between the pH ranges of 7, 8.2 and 9.1.Therefore a pH range from 7 – 9.1 with a thermal stimulation gave the best results.

3.3. Chemical stimuli on the spawning of mussels

3.3.1. Hydrogen peroxide (H_2O_2)

Concentrations of 2 mM and 5mM Hydrogen Peroxide were able to induce partial spawning in *P. viridis*, only after 13 hours. Higher concentrations did not evoke any response rather caused stress in the animals as observed by the heavy mucous production and closing of the shell valves. Percentage of spawning by 5 mM H_2O_2 was twice better than that of 2 mM H_2O_2. The percentage spawning was 16.67 ± 5.77 % at 2mM. While it was 30 ± 10 % at 5 mM. In the control, 66.67 ± 5.77 % spawned (Fig. 10). The percentage of spawning was poor when compared to the controls, where they spawned profusely. Spawning in Hydrogen Peroxide treated mussels was partial in nature and the gametes were not viable.

Mussels under control spawned significantly better than the H_2O_2 induced ones. Percentage of mussels induced to spawn by different concentrations (2mM and 5mM) of H_2O_2 did not differ significantly (Table 11). Overall, control was the best.

3.3.2. Tris- Hydroxymethyl Aminomethane (Tris)

2 mM and 5 mM concentrations of Tris at pH 8.2 was successful to induce spawning in *Perna viridis*. Lower concentrations of 2 mM were successful in inducing partial spawning (Fig. 11), but only after 19

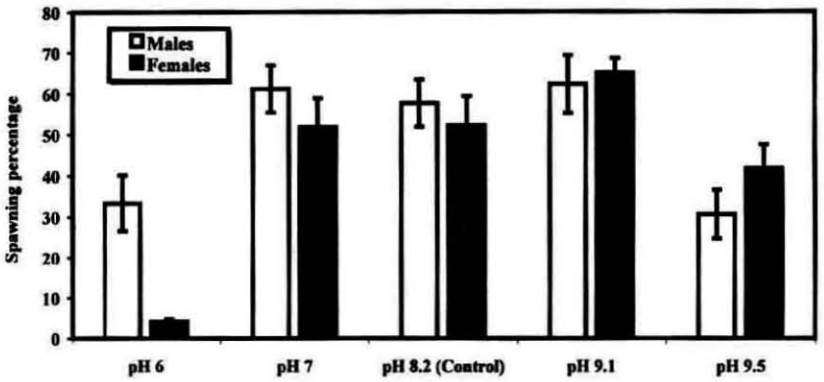


Fig. 8. Effect of pH on the induced spawning of *Perna viridis* (vertical bars represent standard deviation)

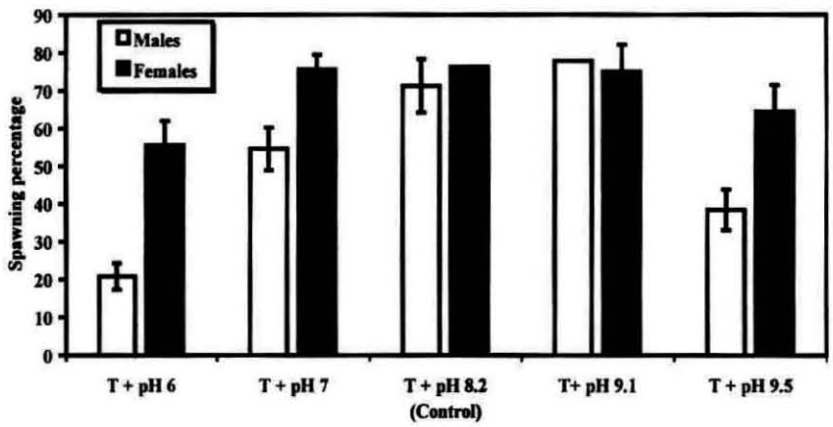


Fig. 9. Combined effect of temperature and pH on induced spawning of *Perna viridis* (vertical bars represent standard deviation)

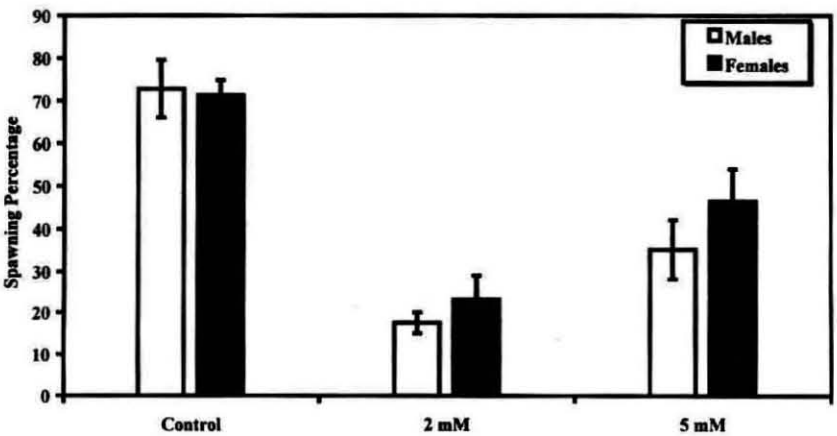


Fig. 10. Effect of different millimolar concentrations of Hydrogen peroxide (H_2O_2) on the inducement of spawning in *Perna viridis* (vertical bars represent standard deviation)

hours. 23.33 ± 5.77 % , 6.67 ± 2.52 % and 66.67 ± 5.77 % spawned in Tris with 2 mM, 5 mM and in the control respectively (Fig. 11). There was no response in animals exposed to Tris pH 8.2 at concentrations of 10 mM, 20 mM and 30 mM even after 24 hours. With Tris, more males spawned than females but the gametes were not viable.

There was significant difference in the spawning percentage between the controls and Tris induced mussels. Percentage of spawning in controls was significantly better than Tris induced spawning ones (Table 11). There was no significant difference between the treatments at different Tris concentrations. Here also, control gave the best result.

Tris 2 mM at different pH and H_2O_2, and 5 mM at different pH adjusted with Tris 2 mM also resulted in partial spawning with non viable gametes.

3.3.3. Serotonin

Serotonin (2 mM) injected into the posterior adductor muscle gave partial spawning after 4 hours. The percentage spawning was 16.67 ± 3.54 %. In the control 66.67 ± 5.77 % spawned after 3 hours (Fig. 12).

A similar dosage added to the water did not invoke any response even after 24 hours. Percentage of animals spawned in

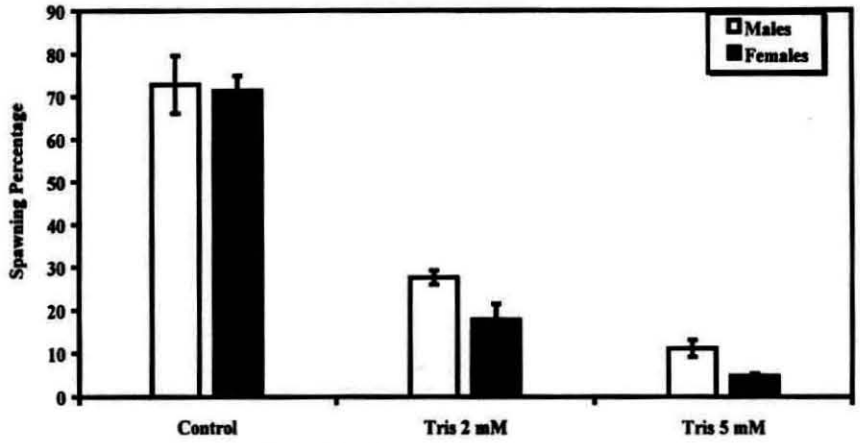


Fig. 11. Effect of varying millimolar concentrations of Tris at constant pH 8.2 on the inducement of spawning in *Perna viridis* (vertical bars represent standard deviation)

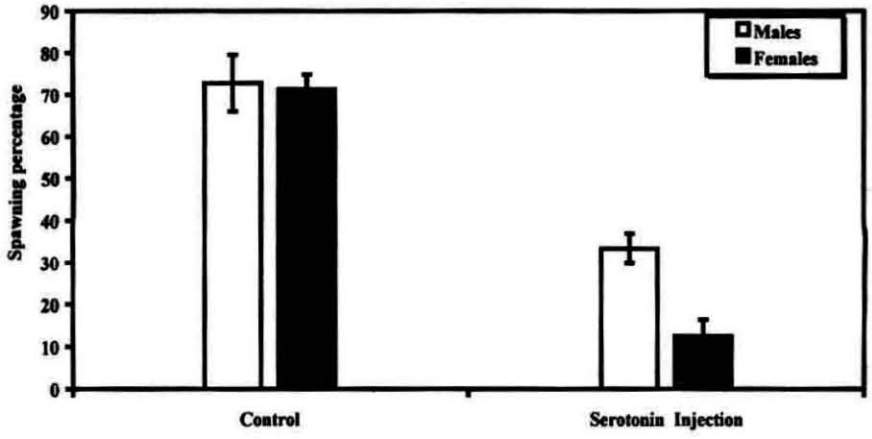


Fig. 12. Effect of 2 mM Serotonin on the inducement of spawning in *Perna viridis* (vertical bars represent standard deviation)

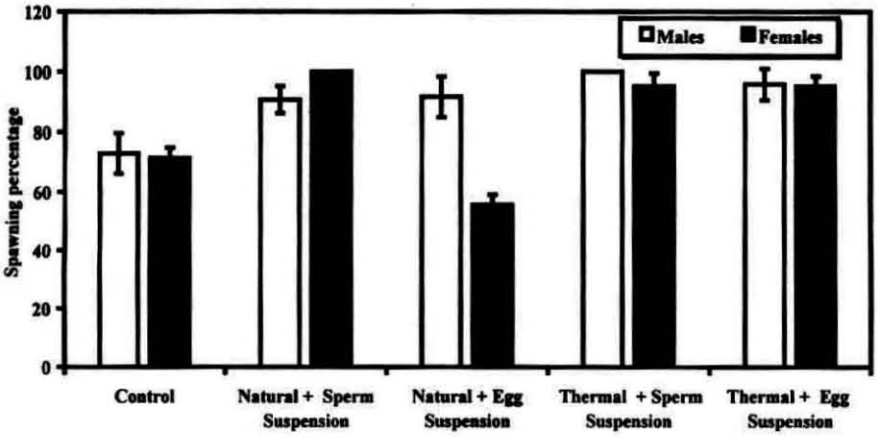


Fig. 13. Effect of gamete addition on the inducement of natural and thermally stimulated spawning in *Perna viridis* (vertical bars represent standard deviation)

controls was significantly superior than that in Serotonin injected animals (Table 11).

3.3.4. Potassium Chloride

Potassium Chloride, in millimolar concentrations either injected or added to the water did not induce spawning in mussels even after 24 hours.

3.4. Biological Stimulation

Effect of gamete addition on spawning induction in cleaned mussels revealed that majority of mussels responded within 2 hours to trigger spawning. The results were better than the control, though the results were not statistically significant (Fig. 13, Table 10).

3.4.1. Natural spawning with sperm suspension addition

Addition of sperm suspension to seawater was very effective to induce spawning in both the sexes (Fig. 13). Overall 93.33 ± 5.77 % spawned (90.74 ± 4.98 % and 100 % in males and females respectively). In the control, 66.67 ± 5.77 % spawned (Fig. 13). Males spawned within 1.5 hours and females after 2 hours.

3.4.2. Natural spawning with egg suspension addition

Percentage spawning was same as the control. 80 ± 5.77 % (91.67 ± 6.75 % males and 55.56 ± 3.53 % females) spawned 2 hours after addition of the suspension, while 66.67 ± 5.77 % spawned in the control (Fig. 13). Males responded within 45 minutes and females after one and a half hours.

Addition of gametes to stimulate spawning neither did significantly increase the spawning percentage of mussels when compared to the controls nor showed any significant difference in the percentage of mussels spawned between the gametes of males or females added to the water (Table 10).

3.5. Combination of Temperature and Biological stimulation

3.5.1. Thermal stimulation with sperm suspension addition

Thermal stimulation with sperm suspension addition was better than controls but only marginally compared to natural spawning combined with sperm suspension addition. (Fig. 13). 96.67 ± 5.77 % (100 % and 95.24 ± 4.25 % males and females respectively) responded to this treatment, while in the control, 66.67 ± 5.77 % spawned (Fig. 13). Males spawned in 2 hours while the females responded after two and a half hours.

3.5.2. Thermal stimulation with egg suspension addition

In the case of egg suspension there was not much difference in the male and female response to spawning. Spawning percentage was only marginally less when compared to sperm suspension results. (93.33 ± 5.77 %) while 66.67 ± 5.77 % spawned in the controls. (Fig.13). Males spawned in 1 hour and females in one and a half hours. The percentage of females spawned was nearly double when compared with natural spawning with egg suspension.

Statistically though the percentage spawning in temperature stimulated mussels with male or female gamete addition was not

significantly different from normally spawned with gamete addition (Table 11). However, the results were significantly better than the control. From the present study it was inferred that temperature stimulation with the addition of male or female gametes was the best treatment for the induction of spawning in *P. viridis* under controlled conditions.

Table 9. Induced spawning of *P. viridis:* Natural spawning

2 - Way Analysis of Variance (2- Way ANOVA)

Source of Variation	SS	df	MS	F	P-value
Sex	465.43	1.00	465.43	0.24	0.63
Treatments	71999.27	15.00	4799.95	2.48	0.006 *
Interaction	16883.48	15.00	1125.57	0.58	0.88
Within	124136.77	64.00	1939.64		

Table 10. Induced spawning of *P. viridis:* Thermal stimulation

Source of Variation	SS	df	MS	F	P-value
Sex	1376.44	1.00	1376.44	3.15	0.08
Treatments	21522.32	14.00	1537.31	3.52	0.00 *
Interaction	2376.89	14.00	169.78	0.39	0.97
Within	26194.39	60.00	436.57		

Table 11. Induced spawning of *P. viridis:* Effect of chemicals

Source of Variation	SS	df	MS	F	P-value
Sex	14.91	1.00	14.91	0.23	0.88
Treatments	38532.23	18.00	2140.68	3.29	0.00 *
Interaction	2376.89	14.00	169.78	0.39	0.97
Within	26194.39	60.00	436.57		

*** significant at $p < 0.05$**

4. DISCUSSION

Desiccation was not a major factor in inducing *P. viridis* to spawn. This contrasts the works of Berg and Kutsky (1951), Scarpa *et al.* (1994 *b*), Toro and Sastre (1995), Kiyomoto *et al.* (1996) who stimulated spawning in *M. edulis*, *M. chilensis*, *M. galloprovincialis* respectively by a combination of desiccation and subsequent exposure to rising temperature. Bayne (1965) and Dixon (1982) both succeeded to induce spawning *M. edulis* by a combination of desiccation, thermal stimulation and injection of KCl into mantle cavity. Roy and Joseph (1992) found the methods of desiccation and thermal stimulation the best for inducing spawning in *P. indica* under controlled conditions. The marginally improved results obtained in this study could be due to the fact that the animals were not subjected to any additional thermal stimulation which could have resulted in more animals to spawn in captivity as done in the other reports.

High salinities have been found to induce spawning in *P. viridis* in the wild from the east coast (Narasimham, 1980; Sreenivasan *et al.* 1989, *b* ; Rajagopal *et al.*, 1997, 1998 *a, b*) and along the west coast (Parulekar *et al.*, 1982 ; Rivonker *et al.*, 1993) of India. There are also reports of other mussel species spawning at high salinities in temperate countries especially *M. edulis* (Bayne, 1976; Innes and Haley, 1977; Sastry, 1979) and clam *Corbicula japonica* (Baba *et al.*, 1999). High salinities have also been reported

as necessary to spawn other bivalves in the wild (Mane, 1974; Nair and Nair, 1985; Kenny *et al.*, 1990; Mann *et al.*, 1994). Sreenivasan *et al.* (1989 *b*) had indicated that *P. viridis* could spawn in less than 30 ppt (maximum salinity drop in that study was found to be to 22.67 ppt and temperature between 29.3 and 32.9 ° C). Hence, it could be concluded that *P. viridis* spawns between 22 – 38 ppt .

However, low salinity or abrupt salinity fluctuations have also been reported to trigger spawning in *P. viridis* (Paul, 1942; Nagabhushanam and Mane, 1975; Qasim *et al.*, 1977; Ramachandran, 1980; Stephen and Shetty, 1981; Lim, 1992; Appukuttan *et al.*, 2001), *P.indica* (Kuriakose, 1973 ; Appukuttan and Nair, 1980, 1983; Appukuttan *et al.*, 1989) and *Xenostrobus securis* (Wilson, 1968, 1969). Salinity fluctuations induced in the lab in *M. californianus, M. edulis, M. viridis* and *P. indica* (Young, 1945; Loosanoff and Davis, 1963; Rao *et al.*, 1976; Sivalingam, 1977; Roy and Joseph, 1992) and in the wild in *M. edulis* (Newell *et al.*, 1982; Fell and Balsamo, 1985; Thorarinsdottir, 1996) have also shown to be ineffective in mussel spawning.

Difference in the spawning period of mussels *P. viridis* and *P. indica* along the west coast as observed by Nagabhushanam and Mane (1975), Qasim *et al.* (1977), Appukuttan and Nair (1980, 1983), Kuriakose (1980), Appukuttan *et al.* (1989, 2001) may be due to influence of salinity acting synergistically with the variations in various other hydrographic conditions. Rao (1951) observed that *C. madrasensis* breeds continuously in marine environment but discontinuously in estuarine environment. He commented that what

constitutes favorable conditions for spawning is not the sudden salinity change but the length of the period of stable salinity preceding the spawning. On the contrary, in the present study sudden salinity drop upto a threshold level of 20 ppt and not less from a constant year round salinity of 35 – 40 ppt. definitely spawned mussels in captivity and the percentage increased when an added thermal stimulation was provided.

Stephen and Shetty (1981) found that rapid salinity changes from 34 - 24 ppt spawned *P. viridis* and *S. cucculata* out of the spawning season but *S. commercialis, P. indicia* spawned only when the salinity was low during monsoon eventhough the same treatment was given. This indicated that salinity could be only one of the factors in spawning *P. viridis* and not the major factor in influencing spawning. The views of the above authors that a sudden salinity drop from stable conditions as an effective signal for an individual in a population to synchronize spawning cannot be agreed to as in the present study where in the mussels spawned at salinities 25 -38 ppt and the percentage of mussels spawned naturally was higher at highest salinity (which was nearly the same as the control) but not significantly higher from 25 ppt onwards but significantly more than 20 ppt. This indicated that *P. viridis* has a threshold limit for spawning and salinity of 25 ppt above is conducive for spawning and rapid salinity change is not that essential a factor for enhanced spawning in mussels as observed by them. Similar views were also expressed by Roy and Joseph (1992) for *P. indica* where, the authors believed that the change in salinity is not necessarily the

most important factor in affecting spawning and further stated that in nature *P. indica* spawns in high saline condition (> 35 ppt) similar to that observed for *P. viridis*.

The combined effect of high temperature and high salinity has a synergistic effect on *P. viridis* spawning. The study indicated that irrespective of the salinity significantly more number of mussels spawned when a thermal stimulation was applied. This fact is in agreement with findings of a similar nature in the wild (Rajagopal *et al.*, 1997, 1998 *a, b*). According to Prasad (1954) intensity of spawning is low during vigorous monsoon months but subsequently increases with weakening of the monsoon and resumption of calm conditions. He further added that temperature and abundance of phytoplankton of the proper species acts as a trigger for spawning, but Rajagopal *et al.* (1998 *a, b*) proposed that reproductive ability is related to temperature and not food or salinity. Hence, salinity may not be that an important factor in spawning of green mussel *P. viridis* especially along the east coast of India but could be a factor to some extent along the west coast. Recently Appukuttan *et al.* (2001) presumed that the decrease in the rainfall intensity which results in the water temperature increase triggers spawning. Sastry (1979) had cautioned that the degree of synchronization of spawning might vary among species and within the population of the same species in different parts of their geographic range.

Temperature stimulation was effective in spawning *P. viridis*. Significantly, the stimulation by thermal means combined with other

factors induced more numbers of mussels to spawn than any factor taken alone.

In temperate regions, mussels of the genus *Mytilus* and *Perna* breed in spring or early summer when the water temperatures are high (Seed, 1976; Innes and Haley, 1977; Kennedy, 1977; Sastry, 1979; Hickman and Illingworth, 1980; Shafee, 1989; Seed and Suchanek, 1992; Snodden and Roberts, 1997; Caceres-Martinez and Figueras, 1998) and in sub- tropical regions the mussels are ripe throughout the year and show opportunistic spawning in relation to sudden climatic change (Lubet, 1981).

The results obtained in this study can also be correlated with the reproductive biology of *P. viridis* occurring in nature in the Indian coastal seas, where these mussels have indicated a prolonged spawning season. On the west coast, *P. viridis* spawns from July to December with peaks in September to November and showed a minor peak from February to March (Rao *et al.*, 1975, 1976). On the contrary along the east coast, Narasimham (1980) reported spawning from December to July – August (when temperatures and salinity were high) with spawning peak in the earlier months whereas Sreenivasan *et al.* (1989 *b*) reported spawning in *P. viridis* from October to April and indicated that spawning takes place for a prolonged period. Parulekar *et al.* (1982) found spawning in *P. viridis* before and after heavy monsoon rains when the temperature and salinity are high. This was confirmed by Rivonker *et al.* (1993) and Rajagopal *et al.* (1997, 1998 *a, b*) with the latter reporting that high temperatures but not salinity trigger spawning in *P. viridis* along the

east coast of India. Paul (1942) noticed that the two peaks of salinity and temperature of the coastal waters correspond to the spawning of bivalve molluscs. On the other hand, the only contradicting observation to these studies was that along on the west coast it was low temperature during the southwest monsoon that acts as a trigger for the spawning in *P. indica* (Appukuttan and Nair, 1980, 1983; Kuriakose, 1980; Appukuttan *et al.*, 1989).

Mussels have also shown to be refractive to temperature stimulation or variations in hatchery conditions. *M. edulis* (Loosanoff and Davis, 1963; Berg and Kutsky, 1951) and *M. viridis* (Sivalingam, 1977) failed to spawn when thermally induced, while Young (1945) categorically stated that spawning in *M. californianus* occurred irrespective of the temperature in controlled conditions. No correlation between temperature and spawning was noticed by some workers for *M. edulis* in natural conditions (Newell *et al.*, 1982; Fell and Balsamo, 1985).

Thermal stimulation has been the commonest method adopted in spawning several species of molluscs particularly those in the temperate and subtropical regions. This method is the easiest and has less side effects than chemical treatments (Ino, 1972). The exact mechanism, which induces spawning in these organisms by thermal stimulation, is not clear. However, it is believed that in thermally stimulated animals there is an increased permeability of the ovarian epithelium, which may temporarily render the cell's interior alkaline thereby facilitating spawning (Iwata, 1951 *d*).

There was an increase in the percentage spawning in mid to higher pH ranges (7 – 9.1). Alkaline pH has been used widely for inducement of spawning in mussel *M. edulis* (Iwata 1951 *a – c*; Sagara, 1958; Bayne, 1965, 1976; Breese and Robinson, 1981; Smith and Strehlow, 1983) while fluctuations of pH have also shown to be having no inductive effect in *M. californianus* (Young, 1945).

Spawning in increased pH through ammonification of seawater by NH_4OH / NaOH has been achieved in bivalves though no specific range has been mentioned (Loosanoff and Davis, 1963). Rao *et al.* (1976) and Schweinitz and Lutz (1976) found only partial success in spawning *M. viridis* and *Modiolus modiolus* respectively in alkaline (using NaOH) sea water medium. Breese *et al.* (1963) also spawned *M. edulis* with kraft mill effluents, which was alkaline.

The spawning of *P. viridis* and other bivalves in a narrow alkaline range may be due to the activation of the reproductive tissues to spawn (Morse *et al.*, 1977 *b*, 1978). Stimulation of ovarian cells is induced by the penetration of cations of small ionic diameter such as NH_4, Ba or by the penetration of NH_4OH (Morse *et al.*, 1978). When the interior of the cells become alkaline spawning is induced. Further temperature stimulation increased the proportion of animals to spawn in *P. viridis* may be due to increased permeability of the ovarian epithelium, which may render the interior of the cells temporarily alkaline thereby inducing the discharge (Iwata 1951 *a – d*).

Hydrogen peroxide was able to stimulate only partial spawning in *P. viridis*. This is in partial agreement with the work of Morse *et al.* (1977 *b*, 1978), who found low concentrations of 5 mM H_2O_2 induced synchronous spawning in both males and females of *M. californianus* and *M. edulis* at high pH of 9.1. Morse *et al.* (1978) further stated that bivalves were more sensitive to Hydrogen peroxide concentration than abalones and a concentration of 2 – 5 mM H_2O_2 appeared optimal for the induction of spawning in bivalves in contrast to 5 – 8 mM for abalones. Alagarswami *et al.* (1983) in a series of controlled spawning experiments with Indian pearl oyster *Pinctada fucata* noted that spawning response to H_2O_2 treatment was not quite satisfactory and concentrations of 3 – 6 mM peroxide evoked partial spawning response with pearl oyster as observed in the present study. The response too was after 3 – 4 hours in *M. edulis* and *M. californianus* (Morse *et al.*, 1977 *b*, 1978) and 2 – 6 hours for *P. fucata* (Alagarswami *et al.*, 1983) while it took 13 hours in *P. viridis.* However, it is not known whether viable gametes were produced in these studies (Morse *et al.*, 1977 *b*, 1978).

Hydrogen peroxide in high alkaline pH of 9.1 (made with 2 mM Tris) also was able to initiate partial spawning in *P.viridis*. High alkaline pH 9.1 (with Tris buffer) in combination with 5 mM H_2O_2 was successful in the spawning of the mussels *M. edulis* and *M. californianus* (Morse *et al.*, 1977 *b*) while Alagarswami *et al.* (1983) got contrasting results in *P. fucata* with 3.064 mM H_2O_2 at pH 9.1 and 6.128 mM at NaOH pH 9.1 in 4 – 8 hours in sharp contrast to the spawning response time of 14 – 19 hours in *P. viridis*. Trevelyan and

Chang (1983) induced *M. californianus* to spawn at pH 9.1 in a very high molar strength (six times higher than the present study) 30 mM H_2O_2. Tris buffer at alkaline pH was also induced only partial spawning *P. viridis* within 10 hours of treatment but the gametes were not viable.

Morse *et al.* (1977 *a*, 1978) indicated that spawning in molluscs might result from a peroxide – induced stimulation of the endogenous enzymatic synthesis of potent hormone like prostaglandin molecules. They found that alkaline medium (either made with Tris or NaOH) though was not essential for the peroxide activation and induction of spawning it increases the proportion of animals that will spawn. However, they had not given the percentage of animals spawning in an alkaline medium. Further, there were no specific requirements of Tris, because Sodium Hydroxide was as effective as Tris in the induction of spawning in an alkaline medium. Trevelyan and Chang (1983) observed that there was no added advantage of using elevated pH when a combination of 30 mM H_2O_2 treated animals with mechanical stimulation.

Serotonin was found to induce spawning only partially in injected males in this study. Whereas Tan (1997) obtained profuse spawning in the same species and Ram *et al.* (1993), Fong *et al.* (1996) and Fong (1998) for *Dreissena polymorpha*. The positive response of males over females is in agreement with the findings of Gibbons and Castagna (1985) who reported males of *M. mercenaria* are seven times more likely to spawn when compared to females upon injection with Serotonin into the anterior adductor muscle.

Moreover, injections to anterior adductor muscle of the hard clam induced significantly more numbers to spawn than intragonodal injection. In *P. viridis*, injections were given in the posterior adductor muscle and attributed the resultant spawning in *P. viridis* might not have been due to Serotonin alone but could be attributed to mechanical stimulation of pricking the adductor muscle as well with the injection needle.

The best results were obtained when nearly 100 % of the mussels were able to spawn in the presence of gamete addition with thermal stimulation. Natural spawning of bivalves have been achieved through addition of gametes especially, sperm suspension. In mussels, the results of biological stimulation have been mostly negative (Loosanoff and Davis, 1963; Bayne, 1965; Culliney, 1971; Rao *et al.*, 1976; Schweinitz and Lutz, 1976; Sivalingam, 1977; Roy and Joseph, 1992) while other workers (Miyazaki, 1935; Young, 1945; Chipperfield, 1953; Lubet, 1959; Stephen and Shetty, 1981; His *et al.*, 1989) have obtained positive response in mussels.

In *M. edulis* and *M. galloprovincialis,* males can be stimulated to release gametes by eggs or egg extracts but fresh sperm or sperm extracts are not effective in triggering the release of eggs by the female. Young (1945) has also shown evidence that joint action of male and female gametes is more effective than each alone and no difference is shown between sexes as observed in this study on the spawning stimuli.

The presence of gametes along with temperature stimuli increased the proportion of *P. viridis* that spawned. This observation is in agreement to views of Stephen and Shetty (1981) that individuals in the pre - spawning condition tend to withhold spawning and conspecific gametes are the final stimuli even though they observed the reverse in *P. indica*.

CHAPTER III

PHYSICO - CHEMICAL FACTORS AFFECTING *PERNA VIRIDIS* LARVAL REARING

1. INTRODUCTION

Several investigations have been carried out to elucidate the role of physico – chemical factors like salinity, temperature in mussel larval rearing under natural conditions (Bayne, 1976; Lutz and Kennish, 1992), and under controlled conditions (Bayne, 1976, 1983; Widdows, 1991; Lutz and Kennish, 1992). A perusal of literature reveals that two types of experiments were carried out namely, monofactorial experiments (Loosanoff and Davis, 1963; Bayne, 1965; AQUACOP, 1979, 1983; Sprung 1984 *a, b*; Sreenivasan *et al.*, 1988 *a, b*; Satuito *et al.*, 1994; Tan, 1997) and multivariate experiments (Bayne, 1983; His *et al.*, 1989).

Effects of salinity on the larval rearing of mussels have been studied in detail by Young (1941,1946), Hrs-Brenko (1973 *b*) and Tan (1997). The combined effects of salinity and temperature on larval rearing of mussels have been studied by Hrs-Brenko and Calabrese (1969), Lough and Gonor (1973), Hrs-Brenko (1974 *a*, 1978), Lough (1974, 1975) Siddall (1979 *a, b*, 1982), Saranchova and Kulakovskii (1982 *a ,b*), Falmagne (1983), Yaroslavisteva *et al.* (1986) and His *et al.* (1989) .

Several authors have investigated the effects of temperature on mussel larvae in the laboratory (Bayne, 1965; Hrs-Brenko, 1973 *b*; Beaumont and Budd, 1982; Falmagne, 1983; Sprung, 1984 *a* ; Pechenik *et al.*, 1990).

Bayne (1965) reported that agitation or aeration was not necessary for *Mytilus edulis* larvae if water is changed regularly. Earlier experimental studies on mussel larval rearing were conducted in static water systems (Bayne 1965; Rao *et al.*,1976; Schweinitz and Lutz, 1976), and subsequent studies have been conducted in artificially aerated conditions in small containers or big tanks (Tan, 1975 *a*; Siddall, 1979 *a, b* , 1980, 1982; AQUACOP, 1979, 1983; Riisgard *et al.*, 1980; Rengarajan, 1983 *a*; Trevelyan and Chang, 1983; Appukuttan *et al* ., 1984, 1988; Taylor and Beattie, 1985; Eyster and Pechenik, 1987; Sreenivasan *et al.*, 1988 *a, b*; Mosquera *et al.*, 1992).

Very few studies exist on the effective use of antibiotics in mussel larviculture, even though there are numerous reports on its usage in routine larval rearing systems (Le Pennec *et al.*, 1973; Le Pennec and Prieur, 1977; AQUACOP, 1979, 1983; Martin, 1979; Siddall, 1979 *a, b*, 1980, 1982; Beaumont and Budd, 1982; Appukuttan *et al.*, 1984, 1988; Satuito *et al.*, 1994, 1995,1997, 1999)

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Microalgal diets have been used in many studies as tools to assess the nutritional requirements of bivalve larvae. Studies have identified some algal species that can be relied upon to support

mussel and other bivalve larval growth. At present there are several species of phytoplankton being utilized as food sources in various hatcheries and research laboratories worldwide namely *Isochrysis galbana, Monochrysis lutheri, Chaetoceros calcitrans, Tetraselmis suecica, Skeletonema costatum, Pavlova lutheri, Dunaliella* sp. and *Thalassosira pseudonana* (Loosanoff and Davis, 1963; Walne, 1974; Bayne, 1976,1983; Lucas, 1982; Brown *et al.*, 1997; Duerr *et al.*, 1998; Knauer and Southgate, 1999).

3. MATERIAL AND METHODS

3.1. Experimental protocol

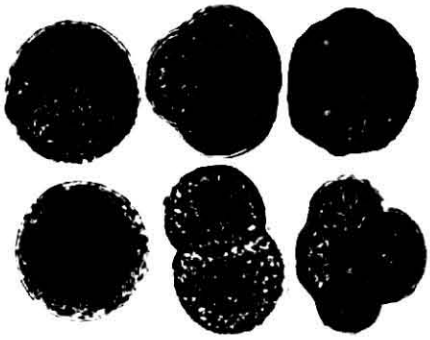
The studies on the early development and larval rearing of the green mussel *Perna viridis* were conducted at Tuticorin Shellfish hatchery of C.M.F.R.I from August 1998 to October 1999 through a series of experiments. Cleaned ripe mussels collected from Pondicherry and Ennore (Chennai) and transported to Tuticorin by road were used for all the experiments.

For spawning experiments 40 green mussels (*Perna viridis*) each were used and in all the cases mussels spawned naturally without any inducement or stimulation. The larval development, sampling and estimations were all done as per the protocols of Loosanoff and Davis (1963) with some minor modifications. For the experiments on the effects of salinity, feed, temperature, antibiotics and pH the larvae were reared in 5 l glass beakers with 4 l sea water, while experiments of aeration and feed were done in 100 l FRP tanks (PLATE VI A) with 80 l sea water. All the experiments were conducted in triplicates. Beakers and tanks were kept covered with a black cloth, to avoid dust and debris falling into them and also to prevent the development of algae, which can interfere with the results.

Twenty eight hours after fertilization the 'D' shaped veliger larvae (PLATE V) were collected by passing the contents through a 100 µm sieve thereby retaining them but, at the same time permitting

PLATE V

Life cycle of *Perna viridis*



Embryonic stages (x400)

Trochophore (x400)

Adult

Veliger (x200)

Spat (x40)

Early Umbo (x200)

Plantigrade (x40)

Late Umbo (x200)

Pediveliger (x40)

Eyed stage (x40)

only the eggs, embryos and trochophore larvae (PLATE V) to pass through. After estimation of the larval density, the desired number of larvae (@ 3 larvae ml $^{-1}$) were introduced into each culture beaker/ tank (for feed experiment only @ 2 larvae ml $^{-1}$). All the larvae were taken from the same spawning stock with an average shell length (anterio posterior measurement) of 82.258 ± 4.12 µm and shell width (dorso ventral measurement) of 69.102 ± 3.06 µm on its breadth. In each experiment, the growth, percentage survival and settlement (only for antibiotic experiment) were noted and recorded as mean ± S.D. No aeration or mechanical agitation of the water was provided.

3.2. Water quality parameters

The ambient water quality parameters recorded during the different experimental trails were as follows. Air temperature: 35 ± 1° C (23 ± 1 ° C in A.C.room), water temperature: 29 ± 1° C (23 ± 1 ° C in A.C.room), salinty 37 ±1 ppt, pH 8.2 ± 0.1, Dissolved Oxygen, 4.8 ± 0.3 ppm, Ammonia : 0.001 ± 0.0015 ppm.

3.3. Feeding protocol

The larvae of green mussel *Perna viridis* were fed anexic unicultures of microalga *Isochrysis galbana*. The microalgae, whose cell concentration was determined by counting a sub sample using a haemocytometer, were fed @ 5000 cells / larvae from the veliger stage to the umbo stage (PLATE V), 8000 cells / larvae from umbo to eyed stage (PLATE V) and 10000 cells / larvae from eyed to pediveliger stage (PLATE V). From pediveliger stage through to the

plantigrade (PLATE V) and spat stage (PLATE V) the feeding rate was 15000 cells / larvae. The required quantity of feed taken from freshly harvested cultures from the algal culture room, was acclimatized to the ambient water temperature conditions, passed through a 40 µm sieve and poured uniformly into the larval rearing tank / beaker just after release of larvae.

3.4. Effect of physico chemical parameters

3.4.1. Salinity

Salinity of 5 ppt range was made from 10 to 30 ppt by diluting fresh filtered seawater of ambient salinity 38 ppt (control) with filtered dechlorinised tap water. The feed prepared in ambient salinity when added to the beakers of lower salinity with larvae, necessary quantity of dechlorinated tap water was added to adjust the overall salinity in each salinity treatment. The experiment was terminated at settlement of spat in all salinities.

3.4.2. Temperature

P. viridis larvae were reared till settlement in temperatures 24 ° C, 27 ° C, 29 ° C (control) ,31 ° C, 33 ° C and 35 ° C which was kept constant using a Jumo thermometer and immersion heater with relay set up (PLATE VII A). The rest of the experimental protocol was similar to the one conducted for the salinity experiment.

3.4.3. Aeration

For this study three different treatments were used *viz*, no aeration (control), heavy aeration and mild aeration. The larval rearing protocol and feeding protocol were similar to the one dealt earlier. Two aerator stones each connected to the main blower line by plastic tubes and regulators were introduced into the rearing tank for aeration. For heavy aeration, the airflow was kept at the maximum by which the rearing water was agitated rapidly. In mild aeration, the water airflow was controlled using regulators so that the water was bubbled.

3.4.4. Antibiotics

Chloramphenicol powder was dissolved in fresh distilled water to get a concentration of 50 ppm in the ambient salinity of 38 ppt. Controls (38 ppt.) were maintained without any antibiotics. The antibiotic was added each day after water exchange and before feeding.

3.4.5. pH

For this experiment, pH 6, 7, 8.2 (control) and 9 was prepared by adding 0.1 N HCl and 0.1 N NaCl to seawater of ambient pH 8.2.The mussel *P. viridis* larval rearing procedures were same as mentioned before. This experiment was conducted for 7 days. Seawater pH was adjusted before adding the feed as it was found that addition of feed did alter the pH in lower pH range only negligibly.

3.4.6. Feeds

The effect of four microlagal feeds on the larval rearing of *Perna viridis* was studied. Pure cultures of *Isochrysis galbana* (Control) *Chaetoceros calcitrans, Chlorella salina., Nanochloropsis* sp. were fed to mussel larvae reared in 100 l FRP tanks. Veliger larvae were stocked @ 2 ml $^{-1}$, for 7 days. The feeding schedule was the same as per earlier mentioned protocols.

3.5. Statistical analyses

Statistical analyses of the actual growth rate data (for all the experiments), arcsine transformed percentage survival data (for all the experiments) and percentage settlement for antibiotics in mussel *P. viridis* larvae were analyzed through a One – way Analysis of Variance (ANOVA) with EXCEL computer software. In all cases, when the F value of the treatments were significantly different, the best treatment was found out through pair wise (Students t – test ($p < 0.05$)) comparison of treatment means.

4. RESULTS

4.1. Salinity

The results of the study indicated that lower salinities of 10, 15 and 20 ppt were lethal for the green mussel *P. viridis* larvae, with total mortality observed on 10^{th}, 18^{th} and 21^{st} days respectively. Consumption of feed was less at low salinities of 10 and 15 ppt, as very little feed was observed in the larval gut under the microscope. First settlement of spat was observed at 25 ppt on day 14 while it took 15 days at salinities 30 and 38 ppt. Spat settlement was completed by day 18 in all salinities (Fig. 14).

The overall growth rate showed a positive trend with increasing salinity. The growth rate was 3.93 ± 1.57, 8.99 ± 0.86, 11.56 ± 0.55, 12.01 ± 0.84, 15.22 ± 2.72 and 19.02 ± 1.68 µm day $^{-1}$ for salinities 10, 15, 20, 25, 30 and control (38 ppt) respectively (Fig. 17). From the 1^{st} to 8^{th} day, the growth rate recorded was 3.93 ± 1.57, 13.88 ± 3.95, 15.98 ± 3.27, 17.02 ± 2.4, 20.69 ± 0.91, 19.12 ± 2.4 µm day $^{-1}$; from 8^{th} – 18^{th} day, it was 7.08 ± 2.4, 8.19 ± 1.1, 10.01 ± 2.91, 11.9 ± 1.3, 19.47 ± 2.8 µm day $^{-1}$ respectively for salinities 15, 20, 25, 30, 38 ppt (control) (Fig. 17). From 18^{th} – 24^{th} day, the growth rate observed was 9.47 ± 1.06, 20.59 ± 3.08 and 23.23 ± 6.43 µm day $^{-1}$ respectively for 25, 30 and 38 ppt (control) (Fig. 17).

It may be noted here that from the 1^{st} – 8^{th} days, the growth rate of the larvae differed significantly only between 38 ppt (control)

and low salinities of 10 and 15 ppt (Table 12). However, from 8^{th} - 18^{th} day growth rates differed significantly between 38 ppt (control) and salinities of 30, 25 and 20 ppt. In 30 and 20 ppt, the growth rate of the larvae did not differ significantly (Table 12). From the 18^{th} – 24^{th} day, except between the 38 ppt (control) and 30 ppt, growth rate in all other salinties differed significantly (Table 12).

Overall growth rate of the larvae reared in different salinity differed significantly with the control (38 ppt). There was also no significant difference between 30, 25 and 20 ppt and between 10 and 15 ppt (Table 12).

Between the 1^{st} –8^{th}, 8^{th} –18^{th} and 18^{th} – 24^{th} days, there was no significant difference in the growth rate of the larvae in 38 ppt (control). Growth rate of larvae reared in 30 and 25 ppt differed significantly only between 1^{st} –8^{th}, 8^{th} –18^{th} days of the rearing cycle. In salinities 20 and 15 ppt, growth rate of the larvae did not differ significantly between the between 1^{st} – 8^{th} and 8^{th} –18^{th} days.

Percentage of larval survival was 13 ± 3 %, 31.67 ± 5.69 %, 35.33 ± 5.03 % at 25, 30 and 38 ppt respectively (Fig.18). There was significant difference in the survival percentage in 25, 30 and 38 ppt while there was no significant difference in the percentage survival in 30 ppt. and control (Table 12).

Thus, salinity range of 30 – 38 ppt was the optimal salinity range for *P. viridis* larval survival while best growth rate obtained was at 38 ppt.

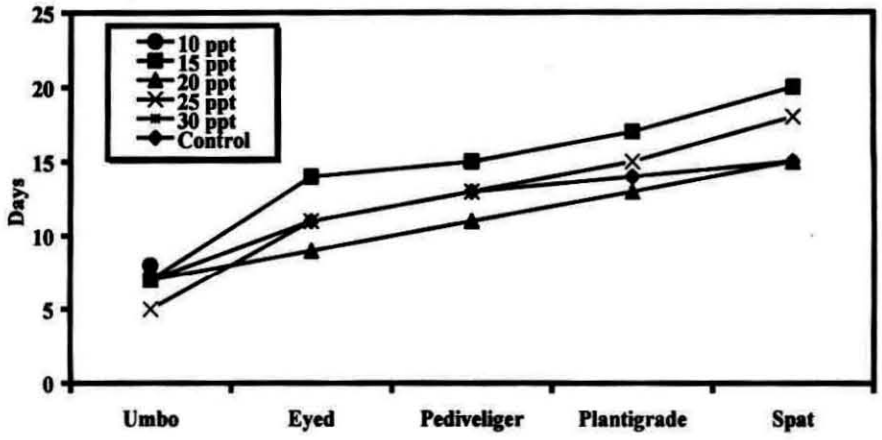


Fig. 14. Days taken to attain different stages in *Perna viridis* larvae grown in different salinity

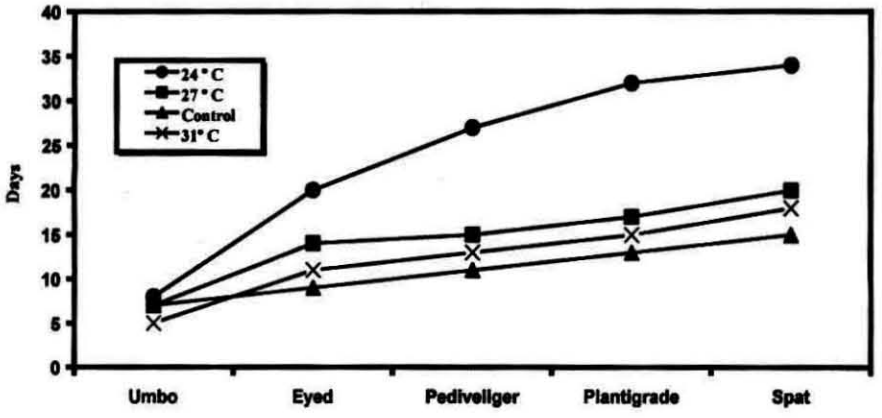


Fig. 15. Days taken to attain different stages in *Perna viridis* larvae grown in different temperature

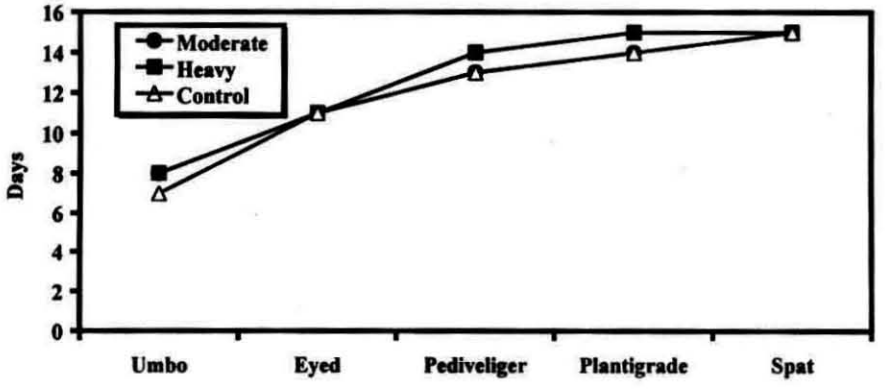


Fig. 16. Days taken to attain different stages in *Perna viridis* larvae grown in different aerated conditions

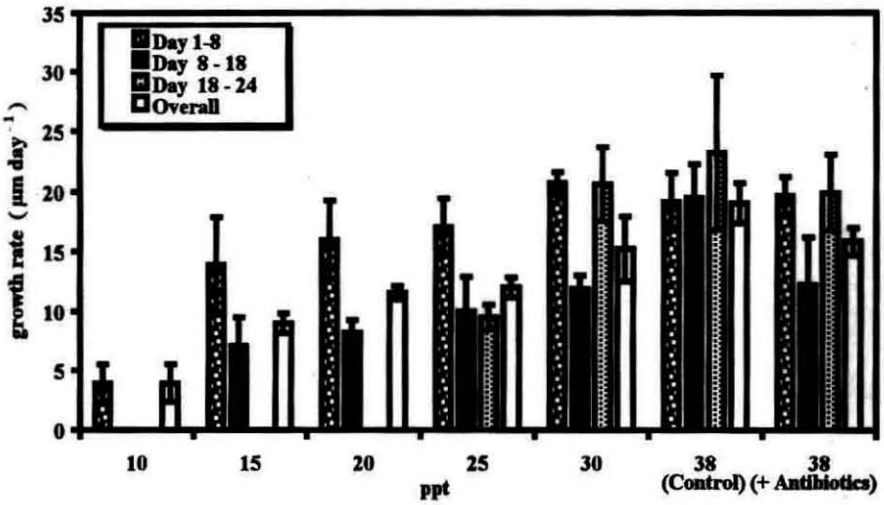


Fig. 17. Growth rate of *Perna viridis* larvae reared for 24 days at different salinities (vertical bars represent standard deviation)

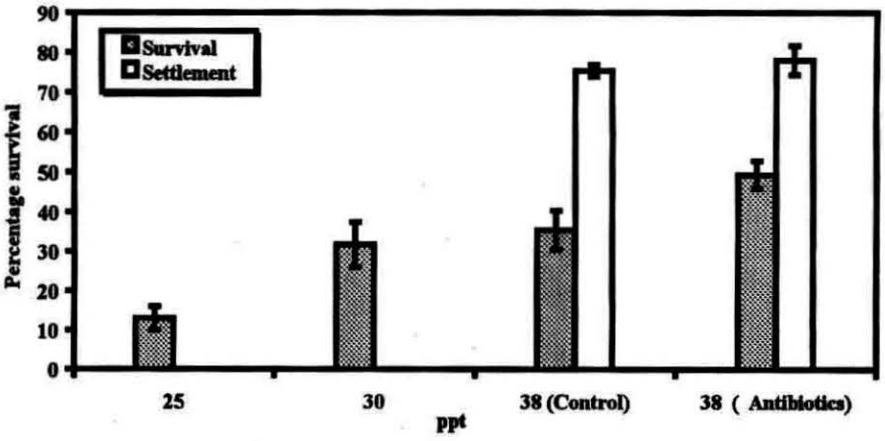


Fig. 18. Percentage survival and settlement of *Perna viridis* larvae reared in different salinities (vertical bars represent standard deviation)

4.2. Temperature

Progressive trend in the developmental stages in *P. viridis* larvae was seen increasing with temperatures from 24 ° C to 31 ° C. There was total larval mortality after 24 hours at 33 ° C and 35 ° C. At 24 ° C, the larval development was prolonged when compared to other temperatures. Spat settlement was first observed on the 15^{th}, 18^{th}, 20^{th} and 34^{th} day for temperatures 29 ° C (control), 31 ° C, 27 ° C and 24 ° C respectively, and settlement was completed on 18^{th}, 21^{st}, 24^{th} and 41^{st} day for temperatures 29 ° C, 31 ° C, 27 ° C and 24 ° C respectively (Fig. 15).

Overall growth rate of the larvae was 6.12 ± 0.19, 10.15 ± 0.895, 13.43 ± 1.38 and 17.09 ± 2.22 µm day $^{-1}$ for temperatures 24 ° C, 27 ° C, 29 ° C and 31 ° C respectively (Fig. 19). From 1^{st} – 10^{th} day the growth rates were 4.6 ± 1.22 , 4.69 ± 4.29 , 18.84 ± 1.87 and 17.21 ± 1.87 µm day $^{-1}$; from the 10^{th} – 21^{st} day, (day up to settlement except for 24 ° C), the growth rate was 8.34 ± 2.77, 11.08 ± 1.68 , 11.7 ± 1.68 and 20.94 ± 4.46 µm day $^{-1}$ respectively in 24 ° C and 27 ° C, 29 ° C and 31 ° C (Fig. 19). The growth in the final phase of the life cycle at 24 ° C (day 21 – 36) was 7.51 ± 0.41 µm day $^{-1}$ (Fig. 19).

Overall growth rate of larvae at 29 ° C (control) did not vary significantly from that at 31 ° C while the growth rate in both these temperatures differed significantly with 24 ° C and 27 ° C. From the 1^{st} – 10^{th} day, growth rate of the larvae at 31 ° C differed significantly with the growth rate at all other temperatures except with that of

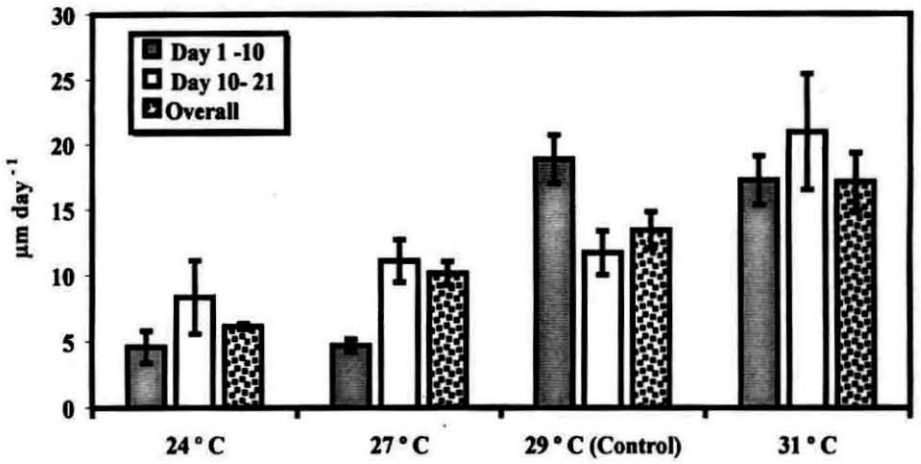


Fig. 19. Growth rate of *Perna viridis* larvae reared in different temperatures (Vertical bars represent standar deviation)

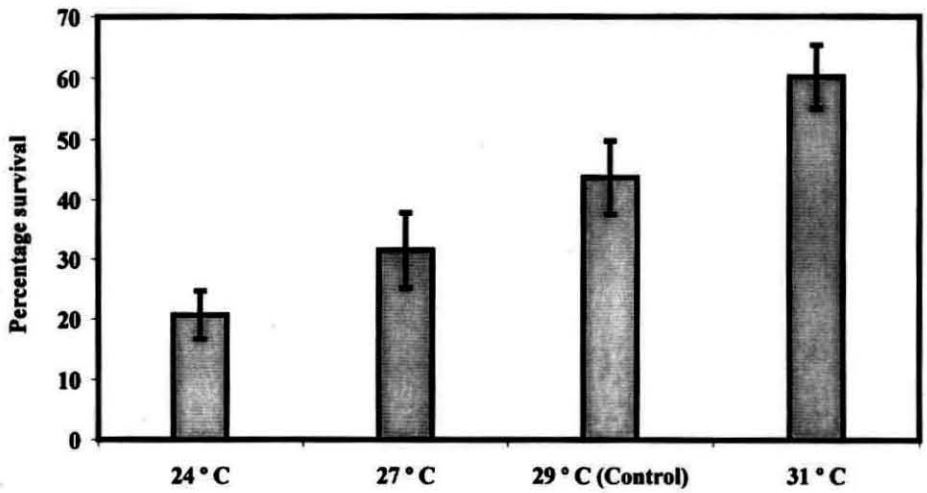


Fig. 20. Percentage survival of *Perna viridis* larvae reared in different temperatures (vertical bars represent standard deviation)

29 ° C. Growth rate at 29 ° C also differed significantly from the growth rates at temperatures 27 ° C and 24 ° C while growth rate at 24 ° C and 27 ° C did not vary. For the second half of the rearing cycle, from the 10^{th} – 21^{st} day growth rate at 31 ° C differed significantly with that of the other temperatures including the control, while growth rates of the larvae at other temperatures (24 ° C and 27 ° C, 29 ° C) did not differ significantly between them (Table 13).

The percentage survival was 20.67 ± 4.02 %, 31.5 ± 6.26 %, 43.67 ± 6.11 % and 60.12 ± 5.19 % at 24 ° C, 27 ° C, control and 31 ° C respectively (Fig. 20). Analysis of variance between the survival percentage in different temperatures indicated significant difference between them (Table 13). Hence, in the mussel *P. viridis* larval survival was best at 31 ° C.

It may be concluded, therefore, that the growth rate of *P. viridis* larvae was best at 29 – 31 ° C range whereas, percentage survival was best at 31 ° C

4.3. Aeration

The number of days required to attain various stages at different conditions of aeration are given in Fig. 16.

Overall growth rate of the larvae reared in varying conditions of aeration showed that non aerated conditions gave the best growth rate followed by moderate and then heavily aerated conditions 18.45 ± 1.27, 17.48 ± 3.88 and 13.81 ± 1.29 µm day^{-1} respectively. Growth rate of the larvae from 1^{st} – 7^{th} day was 15.58 ± 1.29, 16.19 ± 3.88

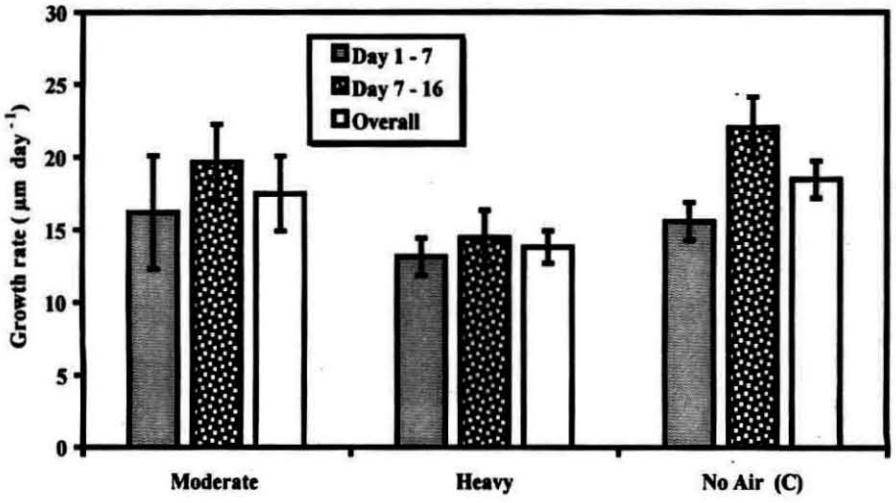


Fig. 21. Growth rate of *Perna viridis* larvae in different aerated conditions (vertical bars represent standard deviation)

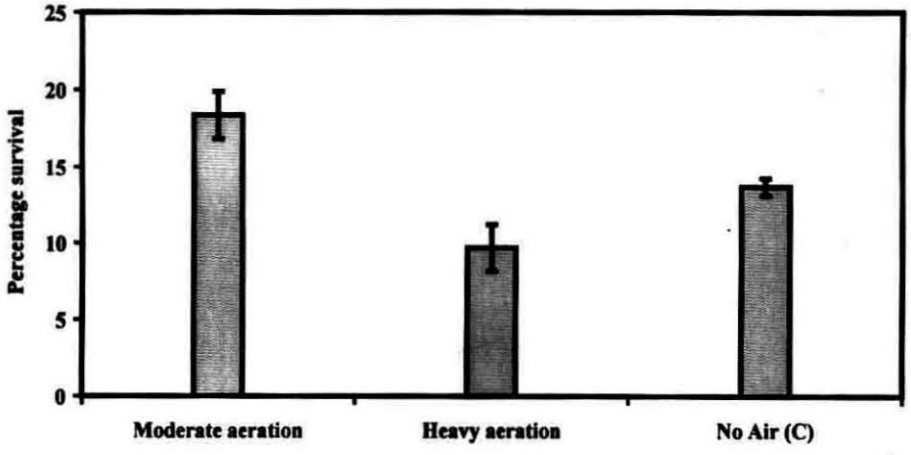


Fig. 22 . Survival of *Perna viridis* larvae in different aerated conditions (vertical bars represent standard deviation)

and 13.14 ± 1.29 µm day $^{-1}$ (Fig. 21) and from the 7th - 16th day the larval growth rate was 22 ± 2.11, 19.64 ± 2.59 and 14.46 ± 1.9 µm day $^{-1}$ in control, moderate and heavily aerated conditions respectively (Fig. 21).

Significant difference existed in the overall growth rate of larvae only between, heavy and non aerated conditions (control) and none between moderate and the control (Table 14). The same was also observed for the growth rate data for 1st -7th day and 7th –16th day. Moreover, no significant difference was observed between the growth rate for 1st -7th day and 7th –16th day in any of the three treatments (Table 14).

The percentage of larval survival was more in moderately aerated 18.33 ± 1.53 % and least in heavily aerated conditions 9.67 ± 1.53 % while 13.67 ± 0.58 % survived in the control (Fig. 22). Significant differences existed in the percentage survival between moderate and heavily aerated and moderate and control conditions. Significant difference was also seen between the heavily aerated and control. It was found that best survival was found in moderate followed by non aerated conditions (Table 14).

Spatfall was first observed in the control on the 14th day, while it took one more day under conditions of moderate and heavy aeration. Spat settlement ended on the 18th, 19th and 20th days in the control, mildly aerated and heavily aerated conditions respectively.

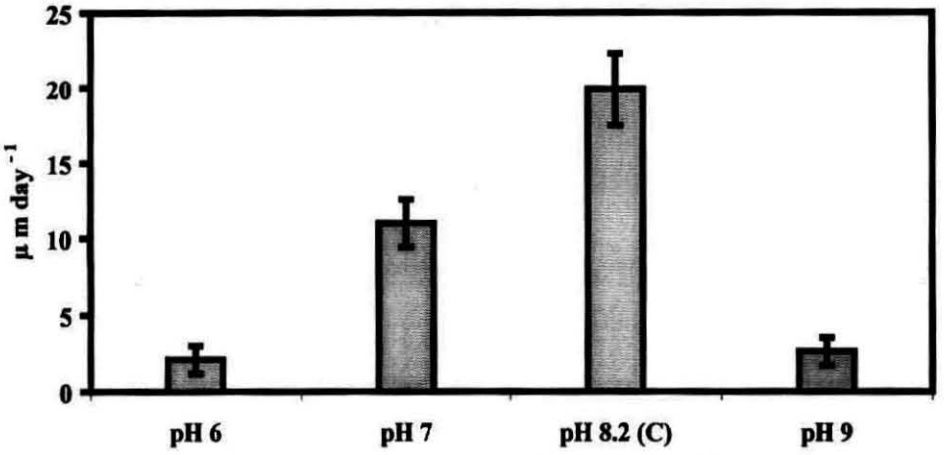


Fig. 23. Growth rate of *Perna viridis* larvae in different pH (vertical bars represents standard deviation)

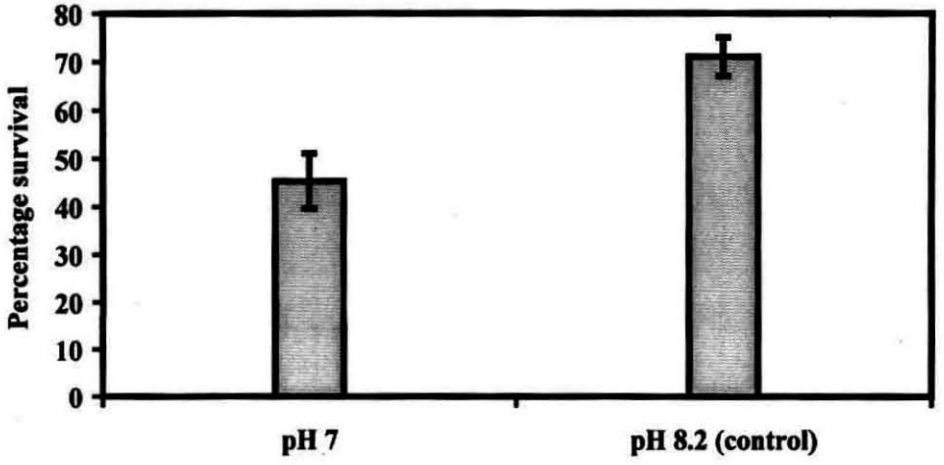


Fig. 24. Survival of *Perna viridis* larvae in different pH (Vertical bars represent standard deviation)

From the present study it was observed that moderately or non aerated (control) conditions was better than heavily aerated condition for the growth of *P. viridis* larvae. Further, it was found that best survival was in moderate aeration followed by non aerated conditions.

4.4. Antibiotic

The time to attain the different larval stages is given in Table 15. Overall growth rate data showed that the larvae reared in antibiotic medium grew at a lesser rate (15.83 ± 1.16 µm day $^{-1}$) than the control (19.02 ± 1.68 µm day $^{-1}$) (Fig 17). Initially from the 1st – 8th day, the growth rate in antibiotic treated cultures was marginally better (19.64 ± 1.57 µm day $^{-1}$) than the control (19.12 ± 2.4 µm day $^{-1}$) (Fig. 17), but subsequently from the 8th – 18th day the growth rate was 19.47 ± 2.8 µm day $^{-1}$ and 12.2 ± 3.97 µm day $^{-1}$, while from the 18th – 24th day it was 23.23 ± 6.43 µm day $^{-1}$ and 19.9 ± 3.18 µm day $^{-1}$ for the control and antibiotic treated larvae respectively (Fig. 17).

On the 24th day, the mean shell length (µm) of the spat reared with antibiotic (395.15 ± 73.43) was less than that of the control (464.2 ± 103.1). In the case of growth rate, there was no significant difference between the control for the overall growth rate and among the days, even though the growth was less than that of the control. Statistical treatment revealed that the overall growth rate did not differ significantly among the control and antibiotic treated larvae and

only significant difference was seen in the growth rate of larvae from the 8^{th} – 18^{th} day (Table 15).

Percentage survival was 49.22 ± 3.39 % in antibiotic administered treatment when compared with the control's 35.33 ± 5.03 %. Settlement percentage was 78 ± 3.61 % when compared with the control 75.33 ± 1.53 % (Fig. 18). Spat settlement began on day 15 and ended on day 19 similar to that of the control.

Percentage survival was significantly better in antibiotic administered treatment when compared with the control while percentage settlement was not significantly better when compared with the controls (Table 15).

4.5. pH

The effect of pH on the mussel *P. viridis* larval rearing indicated total mortality of the larvae at pH 5, 6, and pH 9 in 24 hours, 72 hours and 96 hours respectively. Umbo stage was reached on the 8^{th} day in pH 7 while it reached the umbo stage on the 7^{th} day in pH 8.2 (control).

Overall growth rate of the larvae reared in control pH (8.2) exhibited the best growth rate of 19.9 ± 2.4 µm day $^{-1}$ while those reared in pH 6, 7 and 9 exhibited poor growth rates of 2.095 ± 0.91, 11 ± 1.57, and 2.62 ± 0.91µm day $^{-1}$ respectively (Fig. 23). Overall growth rate was significantly better in the control pH 8.2 (Table 16).

The survival percentage was low in pH 7 (45.33 ± 5.69 %) whereas it was as high as 71 ± 4 % in the pH 8.2 (control) (Fig. 24). Survival percentage was significantly better in pH 8.2 than in pH 7 (Table 16).

Thus, control pH of 8.2 was the best for the growth and survival of *P. viridis* larvae.

4.6. Feed

Ninety percent of the control feed fed larvae reached the Umbo stage and 5 % late Umbo stage (PLATE V) at the end of 7 days. In *C. calcitrans* fed animals, 60 % of the larvae reached Umbo stage, while in the *Nanochloropsis* sp. and the *Chlorella salina* fed larvae only a few reached early Umbo stage (10 % for *Nanochloropsis* and 2 % for *C. salina* fed animals). When the larvae were observed under the microscope, it was seen that control and *C. calcitrans* fed larvae were very active compared to poor activity in *Nanochloropsis* sp. and *C. salina* fed larvae.

Seven day experiments with feeding four different microalgae to the mussel larvae clearly indicated that control feed of flagellate *I. galbana* was definitely best among the feeds, *C. calcitrans* the next best, followed by *Nanochloropsis* sp. and the worst *C. salina* as indicated by the growth rate data of the larvae, which was 17.11 ± 1.06 µm day $^{-1}$ compared to 7.94 ± 2.43 , 4.28 ± 1.06 and 2.4 ± 1.06 µm day $^{-1}$ for (control) *C. calcitrans, Nanochloropsis* sp. and *C. salina* respectively (Fig. 25).

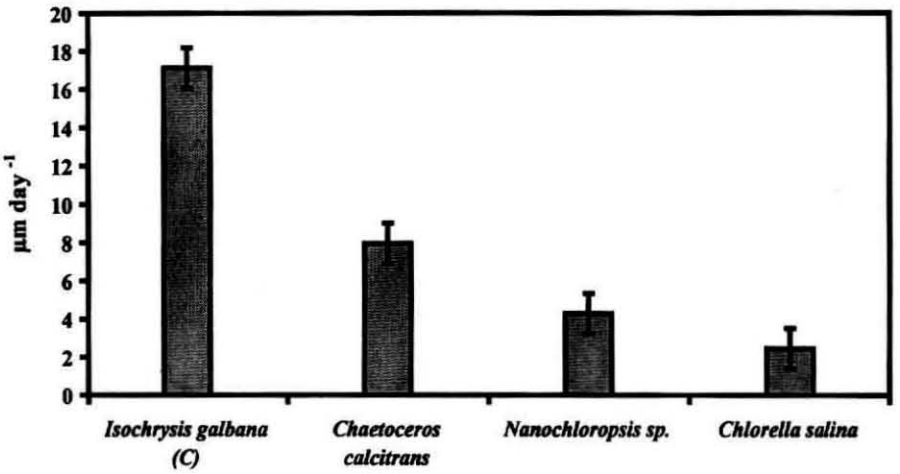


Fig. 25. Growth rate of *Perna viridis* larvae fed with different feeds (vertical bars represent standard deviation)

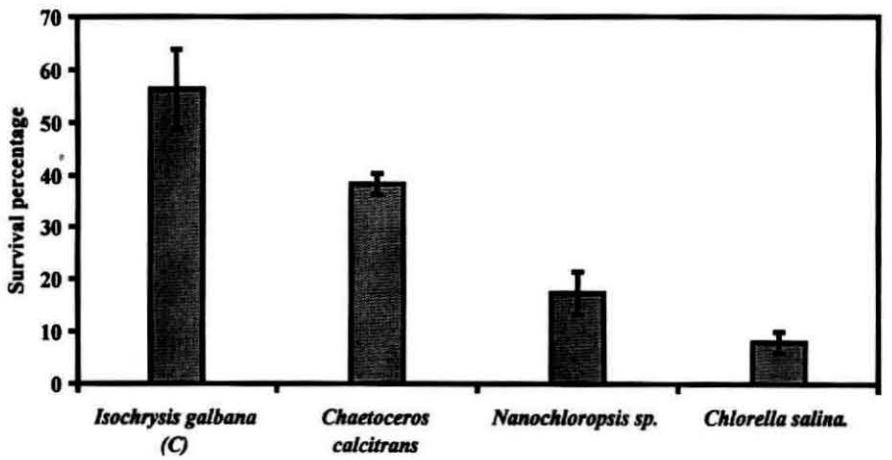


Fig. 26. Survival of *Perna viridis* larvae fed with different feeds (vertical bars represent standard deviation)

Survival rate after seven days also showed the same pattern with the best survival in control with 56.3 ± 7.5 %, *C. calcitrans* the next best with 38 .33 ± 2.08 %, a low of 17.33 ± 4.04 % for *Nanochloropsis* sp. and *C. salina* fed larvae indicated the least survival 8 ± 2 % (Fig. 26).

Growth rate of larvae was significantly better in the control diet than all the other feeds. There was also significant difference between the growth rates of larvae fed with *C. calcitrans* and *Nanochloropsis* sp. and *C. salina* (Table 17). Survival differed significantly among all the treatments with animals fed with control diet showing the best survival than all other feeds (Table 17).

The experiments, therefore, have revealed that *I. galbana* was the best feed for *P. viridis* larval growth and survival.

Table 12. Effect of salinity on *P.viridis* larval rearing
One Way Analysis of Variance (ANOVA)

	Source of Variation	SS	df	MS	F	P-value
Overall Growth Rate	Between Groups	401.55	5.00	80.31	33.35	0.00 *
	Within Groups	28.90	12.00	2.41		
Day 1- 8	Between Groups	534.49	5.00	106.90	15.58	0.00 *
	Within Groups	82.31	12.00	6.86		
Day 8 - 18	Between Groups	288.68	4.00	72.17	14.68	0.00 *
	Within Groups	49.15	10.00	4.92		
Day 18 - 24	Between Groups	319.94	2.00	159.97	9.24	0.00 *
	Within Groups	103.91	6.00	17.32		
Percentage Survival	Between Groups	414.72	2.00	207.36	22.25	0.00 *
	Within Groups	55.91	6.00	9.32		

Table 13. Effect of temperature on *P.viridis* larval rearing
One Way Analysis of Variance (ANOVA)

	Source of Variation	SS	df	MS	F	P-value
Overall Growth Rate	Between Groups	401.55	5.00	80.31	33.35	0.00 *
	Within Groups	28.90	12.00	2.41		
Day 1- 8	Between Groups	534.49	5.00	106.90	15.58	0.00 *
	Within Groups	82.31	12.00	6.86		
Day 8 - 18	Between Groups	288.68	4.00	72.17	14.68	0.00 *
	Within Groups	49.15	10.00	4.92		
Day 18 - 24	Between Groups	319.94	2.00	159.97	9.24	0.01 *
	Within Groups	103.91	6.00	17.32		
Percentage Survival	Between Groups	98.98	1.00	98.98	15.42	7.71
	Within Groups	25.67	4.00	6.42		

Table 14. Effect of aeration on *P.viridis* larval rearing
One Way Analysis of Variance (ANOVA)

	Source of Variation	SS	df	MS	F	P-value
Overall Growth Rate	Between Groups	40.81	2.00	20.40	18.23	0.00 *
	Within Groups	6.72	6.00	1.12		
Day 1- 7	Between Groups	15.69	2.00	7.84	0.20	0.83
	Within Groups	239.76	6.00	39.96		
Day 7 - 16	Between Groups	92.24	2.00	46.12	7.45	0.02 *
	Within Groups	37.15	6.00	6.19		
Percentage Survival	Between Groups	38.79	2.00	19.40	24.87	0.00 *
	Within Groups	4.68	6.00	0.78		

Table 15. Effect of antibiotics on *P.viridis* larval rearing

One Way Analysis of Variance (ANOVA)

	Source of Variation	SS	df	MS	F	P-value
Overall Growth Rate	Between Groups	401.55	5.00	80.31	33.35	0.00 *
	Within Groups	28.90	12.00	2.41		
Day 1- 8	Between Groups	534.49	5.00	106.90	15.58	0.00 *
	Within Groups	82.31	12.00	6.86		
Day 8 - 18	Between Groups	288.68	4.00	72.17	14.68	0.00 *
	Within Groups	49.15	10.00	4.92		
Day 18 - 24	Between Groups	319.94	2.00	159.97	9.24	0.01 *
	Within Groups	103.91	6.00	17.32		
Percentage Survival	Between Groups	98.98	1.00	98.98	15.42	7.71
	Within Groups	25.67	4.00	6.42		
Percentage Settlement	Between Groups	5.13	1.00	5.13	1.43	7.71
	Within Groups	14.33	4.00	3.58		

Table 16. Effect of pH on *P.viridis* larval rearing

One Way Analysis of Variance (ANOVA)

	Source of Variation	SS	df	MS	F	P-value
Overall Growth Rate	Between Groups	443.10	3.00	147.70	1611.43	0.00 *
	Within Groups	0.73	8.00	0.09		
Percentage Survival	Between Groups	343.98	1.00	343.98	40.03	0.00 *
	Within Groups	34.38	4.00	8.59		

Table 17. Effect of feeds on *P.viridis* larval rearing

One Way Analysis of Variance (ANOVA)

	Source of Variation	SS	df	MS	F	P-value
Overall Growth Rate	Between Groups	383.18	3.00	127.73	114.00	0.00 *
	Within Groups	8.96	8.00	1.12		
Percentage Survival	Between Groups	4222.00	3.00	1407.33	69.50	0.00 *
	Within Groups	162.00	8.00	20.25		

* Significant at $p < 0.05$

5. DISCUSSION

P. viridis larvae grew from veliger stage to spat in moderate to high salinity range of 20 - 40 ppt (growth rate better in high salinity) and less than 20 ppt was lethal. This is in agreement with the general observations that growth and survival of mussel larvae are good in a near oceanic seawater (Young, 1941, 1946; Bayne, 1965; Tan, 1975 *a*; Sivalingam, 1977; Hrs-Brenko, 1973 *b*, 1974 *a*, 1978; Hrs-Brenko and Calabrese, 1969; His *et al.*, 1989; Sreenivasan *et al.*, 1989 *a*; Lutz and Kennish, 1992) . It was also noticed in this study that there is a general reduction in larval growth in lower salinities and also heavy mortality in some cases as observed by Hrs-Brenko and Calabrese (1969), Siddall (1979 *a, b*, 1982) and Bayne (1983).

Growth rate of the larvae increased with higher salinity. There was no significant variation in growth in the initial phases from 1st to 8th day, whereas variations were observed only after the 10th day. According to Lough and Gonor (1973) mussel larvae are sensitive to changes in salinity and manifests as reduced growth or mortality at later stages. The mortality of larvae from 15th - 18th days at 15 and 20 ppt may be due to this. Innes and Haley (1977) and Tan (1997) reared larvae of *M. edulis* and *P. viridis* respectively, till settlement. While the former observed no significant decrease in the larval growth rate at different salinities while the latter has reported best growth and survival at 24 ppt. Tan (1997) further explained that larvae developing from embryo to pedeveliger stage, showed a

decrease in tolerance level to lower salinities. He suggested that the larvae of *P. viridis* adjusting to such low salinities may be an adaptation to trigger off spawning during rainy season, whereas Innes and Haley (1977) interpreted the ability of *M. edulis* larvae to grow in low salinities due to the presence of genes influencing larval growth.

There was increased growth, survival and spat settlement of *P. viridis* larvae at 30 and 38 ppt; from broodstock collected or conditioned from areas where salinity was 37 ± 1 ppt. According to Davis (1958) and Downing (1991), larvae of bivalves conditioned at high / low salinities influenced the salinity tolerance of the larvae . Thus the distinct preference of *P. viridis* larvae to higher salinities could be the result of such an acclimatization of the adults.

The larval development was completed in just about 15 days in higher salinites of 30 and 38 ppt, while it took 20 days at 25 ppt at 29 ° C. These results also agree with the observations in temperate species of Bayne (1965), Sprung (1984 *a*), Hrs-Brenko and Calabrese (1969), Hrs-Brenko (1973 *b*) for *M. edulis* (16-20 days), and Hrs-Brenko (1974 *a*, 1978) for *M. galloprovincialis* (20 days) and Rao *et al.* (1976) and AQUACOP (1979, 1983) for *M. viridis* at similar temperature ranges used in this study. However Tan (1975 *a*) and Sivalingam (1977) in *M. viridis*; and Siddal (1979 *a, b*, 1980, 1982) in *P. viridis* reported a shorter period of development. Rengarajan (1983 *a*), Sreenivasan *et al.* (1988 *a, b*), Juario and Benitz (1988), Lim (1992) and Tan (1997) observed slower development for *P. viridis*. Appukkuttan *et al.* (1984, 1988), Trevelyan and Chang

(1983) and Satuito *et al.* (1994, 1995, 1997, 1999) observed the same for *P. indica, M. californianus, M. galloprovincialis* and *M. edulis galloprovincialis* respectively.

In *P. viridis*, the percentage settlement was maximum at the highest salinity and decreased progressively with decrease in salinity. Hrs-Brenko and Calabrese (1969) and Siddall (1979 *a, b*) were of the opinion that variations in salinity tolerance of mussel larvae might be due to the differences in the geographic location and also due to the interactions of other water quality parameters like temperature and food. His *et al.* (1989) through multifactoral experiments on combined effects of salinity and temperature on the growth of mussel larvae *M. galloprovincialis* observed that significant interactions occur only at the extreme limits, and within the salinity range of 20-35 ppt, and concluded that growth is dependent on temperature and feed.

Results of the experimental larval rearing of *P. viridis* at six different temperatures in the present study clearly indicated a direct relationship between temperature on larval growth and survival. There was complete mortality of the larvae reared at 33 ° C and 35 ° C showing that extreme high temperature is not favorable for larval rearing of *P. viridis.* The bivalve larval growth rate increases with increase in temperature to an optimum and then decreases (Bayne, 1983; Widdows, 1991). Similar increase of growth rates with increased temperature has been observed in *P. viridis* (Siddall, 1979 *a, b*). Bayne (1965), Hrs-Brenko (1973 *b*), Trevelyan and Chang (1983), Sprung (1984 *a*), His *et al.* (1989) for *M. edulis*,

M. californianus and *M. galloprovincialis*. On the contrary low temperature also has been reported to be conducive for the growth and survival of *M. galloprovincialis* larvae (Hrs-Brenko, 1974 *a*, 1978; His *et al.*, 1989). The increased growth and survival of bivalve larvae as observed in this study at higher temperatures may be due to better assimilation of enzyme for feed digestion as reported by Loosanoff and Davis (1963) for other bivalve larvae. Robert *et al.* (1988) reported that apart from nutrition, temperature was the dominant factor influencing the growth of bivalve larvae.

Higher temperatures (above 33 ° C) led to total mortality in *P. viridis*. Bayne (1965) also observed mortality of *M. edulis* larvae at 30 ° C and attributed this to the death of algal cells at higher temperature, leading to a bacterial build up in the rearing system. There are no reports of larvae surviving beyond 32 ° C. Davis and Calabrese (1964) found that larvae of *C. gigas* grew well and set at high temperature but growth was reduced at 33 ° C and all the larvae were dead at 35 ° C.

P. viridis larvae are sensitive to low temperature (24 ° C) also which was indicated by poor growth and survival. It took 34 - 45 days for complete settlement in the present study. The slow growth and survival obtained in the present study at 24 ° C in *P. viridis* contradicts the results obtained by Tan (1975 *a*) and Rao *et al.* (1976) for *M. viridis* who were able to settle the larvae within 8-12 and 16 - 19 days respectively in a similar temperature range.

P. viridis larvae preferred moderately aerated or static water conditions, which is in partial agreement with Loosanoff and Davis (1963) and Bayne (1965). Moderate aeration has been routinely used in mussel larviculture of *P. viridis, P. indica, M. viridis, M. edulis, M. californianus* and *M. galloprovincialis* though its effects have not been mentioned (Tan, 1975 *a*; Siddall, 1979 *a, b*, 1980, 1982; AQUACOP, 1979, 1983; Rengarajan, 1983 *a*; Trevelyan and Chang, 1983; Appukuttan et *al.*, 1984, 1988; Taylor and Beattie, 1985; Eyster and Pechenik, 1987; Sreenivasan *et al.*, 1988 *a, b*; Tan, 1997).

The growth rate of *P. viridis* larvae differed significantly between the mildly, heavily aerated and non aerated conditions. On the other hand differences in larval growth have been reported by Helm and Spencer (1972) and Dharmaraj and Shanmugasundaram (1999) who reported significant larval growth differences in aerated and non aerated conditions after day 10 and 7 for *Ostrea edulis* and *Pinctada fucata* respectively. The latter has also reported better growth rate in non aerated conditions when compared with moderately and heavily aerated conditions as observed in *P. viridis*.

The results of aeration on larval rearing in this study contradict the views of Dharmaraj and Shanmugasundaram (1999) who were of the opinion that the necessity of agitating the rearing medium for a particular species of larvae was based on their ecological adaptations. They were of the views that as mussels inhabit shallow waters, the larvae are able to adjust to the prevailing rough sea conditions. Helm and Spencer (1972) were also of the

opinion that aeration of culture may be beneficial to the larvae as it helps in evenly distributing the food. Further, the energy conserved by the larvae, due to their passive maintenance in the water column by turbulent currents, can be used for building up of food reserves and there by body tissues. Mussels, like most other bivalves, prefer a static environment for better growth but from the point of view of survival and settlement, moderate aeration is necessary. Static condition may be better for larval growth but this could lead to accumulation of toxic metabolites, which otherwise would have been oxidized if aeration is provided (Walne, 1966; Helm and Spencer, 1972).

The addition of chloramphenicol to the larval rearing medium of *P. viridis* has significantly improved the survival and settlement percentage. This phenomenon has been reported by many workers for the larvae of mussels and other bivalves. The use of chloramphenicol in larval rearing of mussels *P. viridis, P. indica*, *M. viridis, M. edulis*, *M. californianus, M. galloprovincialis* has been reported by a number of workers (Le Pennec *et al.*, 1973; Le Pennec and Prieur, 1977; AQUACOP, 1979, 1983; Siddall, 1979 *a, b*, 1980, 1982; Appukuttan *et al.*, 1984, 1988; Satuito *et al.*, 1994,1995,1997,1999).

Results of the present study also showed that the antibiotic treated larvae grew well and showed increased growth rate in the early part of the rearing cycle (1^{st} - 8^{th} days) and decreased growth rate thereafter (inferior when compared with the control). Inhibition of larval growth in cultures treated with antibiotics has also been

reported for other bivalve larvae like *Ostrea edulis, Crassostrea rhizophorae, C. virginica, C. gigas, Pinctada fucata* (Walne, 1958; Lemos *et al.*,1994; Le Pennec, 1997; Dharmaraj and Shanmugasundaram, 1999). The decreased growth of the larvae after the 8th day is in agreement with the observations of Lemos *et al* .(1994) who reported that larvae of oyster *C. rhizophorae* grew well up to day 7 and thereafter showed a decrease.

Reasons for the increased growth rates initially and the subsequent growth depression after day 8 of rearing observed in the present study and that reported by Lemos *et al.* (1994) are not known. However, Kinne (1970) cautioned that though antibiotics were beneficial to some extent for larval growth, they might interfere with the other biological processes of the larvae and thereby modify or reduce resistance. Thus the reduced growth rate in the latter part of the rearing cycle in antibiotic treated larvae in the present study could be the result of the larvae becoming weak due to immunosuppression (Walne, 1958; Ukeles, 1975; Fitt *et al.*, 1992).

P. viridis larvae grew best in alkaline pH of 8.2 while, total mortality was observed in pH 5, 6 and 9 within 96 hours. Growth and survival were best in pH of 8.2 when compared to pH 7, similar to that reported by Sivalingam (1977) in *M. viridis*. In contrast, Bayne (1965) stated that veliger larvae of *M. edulis* tolerated low pH but were unable to survive in pH 8.5, pH less than 7 was good for growth and settlement of larvae, and pH greater than 8.3 led to total mortality.

Similarly, pH of 7 to 8.5 have been found to be good for the growth and survival of *Mercenaria mercenaria*, *Crassostrea virginica*, *Mulina lateralis* and *Scapharca broughtonii* (Calabrese and Davis, 1966, 1970; Calabrese, 1970; Wang *et al.*, 1993).

Two reasons have been cited for the reduced growth of bivalves in very low and very high pH. According to Joseph (1983) the reduced growth of bivalve larvae at very low and high pH is mainly because that the microalgae fed to the larvae cannot tolerate very high / very low pH ranges. However, Calabrese and Davis (1966, 1970) contradicted this view by stating that algal cells remained unaffected by pH change. This fact was confirmed in the present study, where in *I. galbana* was found in the gut of the larvae reared in pH levels of 6 and 9 but none in pH 5. Another view by Davis and Calabrese (1964) is that the ciliary activity of bivalve larvae reared in low pH ranges was inhibited thereby affecting food ingestion.

Mussel larvae of *P. viridis* fed with *I. galbana* (5 -10 cells μl^{-1}) showed better growth rate (17 ± 1 μm day^{-1}) and survival, followed by *C. calcitrans*. *Nanochloropsis* sp. and *Chorella salina* did not promote good growth of the larvae. The results were similar to those observed by Bayne (1983) and Widdows (1991) for other mussel larvae. On the contrary, reports indicate poor performance of *I. galbana* when compared with other flagellates or diatoms for the growth and survival of mussel larvae. Among other flagellates found to be more conducive for larval growth and survival are *Pavlova* and *Dicrateria* sp. for both *P. viridis* and *M. edulis*

(Sreenivasan *et al.*, 1988 *b*; McAnally Salas *et al.*, 1992); *Monochrysis* (Bayne ,1965), *Platymonas* (*Tetraselmis*) (Loosanoff and Davis, 1963; Rao *et al.*, 1976) for *M. edulis* and *M. viridis* ; and *Chlamydomonas* (Hirano and Oshina 1963; Zong-Qing and Mei-Fang, 1980) for *M. edulis.* Mosquera *et al.* (1992) found *Chaetoceros* spp. more suitable than *I. galbana* for the rearing of *M. galloprovincialis.*

Growth rates observed for *P. viridis* at higher feed concentration is similar to those obtained by Sreenivasan *et al.* (1988 *b*) (16.69 /um day $^{-1}$), Sreenivasan *et al.* (1988 *a*) (15.89 /um day $^{-1}$) for *P. viridis,* AQUACOP (1983) (17-20 /um day $^{-1}$), Rao *et al.* (1976) for *M. viridis* (14.14 /um day $^{-1}$) and Appukuttan *et al.* (1988) (14.4 /um day $^{-1}$) for *P. indica.* However, Appukuttan *et al.* (1984) reported higher growth rates (35.4 /um day $^{-1}$) for *P. indica.*

The results of mussel larval growth studies at different cell concentration have been conflicting, with some workers observing enhanced growth (Bayne, 1965) and others observing no effect on growth (Sprung, 1984 *a, b*). While considering the cell concentrations, a concentration of 5000 cells larvae $^{-1}$ (5-10 cells µl $^{-1}$) used in this study was found higher than the optimal concentration mentioned by Baylon (1988) and Sreenivasan *et al.* (1988 *a, b*) but lower than that reported by AQUACOP (1979, 1983) and Siddall (1979 *a, b*, 1980, 1982) for the same species. However, increase of food concentration in excess of 10-20 µl $^{-1}$ had little or no

effect on the growth of *M. edulis* and *Pinctada margaritifera* (Sprung, 1984 *a*; Doroudi *et al.*, 1999).

Thus, algal cell densities should be adjusted based on further studies on optimal clearance rates, (a reduction in clearance rate with increasing algal cell concentration has been reported for the larvae of *Mercenaria mercenaria* and *M. edulis* (Riisgard, 1988, 1991) which can withstand poor water quality due to excess of feed in water and increase in microbial activity.

A number of studies have shown that phytoflagellates especially that T- *I. galbana* and *I. galbana* to be of high nutritional value with high protein content but less lipid than *C. calcitrans* (Brown *et al.*, 1997; Kaladharan *et al.*, 1999). *I. galbana* has been good for the growth of mussel larvae (Loosanoff and Davis, 1963; Bayne, 1976, 1983; Brown *et al.*, 1997) and also they produce very little or no toxic metabolites (Davis and Guillard, 1958). Diatoms of the genus *Chaetoceros* especially *C. calcitrans, C. gracilas* and *C. mulleri* have also been used as bivalve larval food in concentration with good results (Helm and Laing, 1987). The reasons attributed to this phenomenon by them were that *Chaetoceros* spp. of diatoms have high Eicosapentaenoic acid (EPA, 20: *5 n*-3) content which is essential for the larvae, while it is deficient in *I. galbana* and T- *I. galbana*.

Laing and Earl (1998) found that the main requirement at spatfall is that eyed larvae should have accumulated adequate lipid reserves though this was not linked to the speed of larval growth.

The type and the amount of biochemical reserves accumulated during larval development with different diets can affect the larval competency to settle (Haws *et al.*, 1993; Baker, 1994). Off late Jonsson *et al.* (1999) found that larval growth and settlement was significantly correlated to three Omega – 3 polyunsaturated fatty acids (PUFA's) (C 18:3, C 18:4 and C 22:6) in the diets. Thus the qualitative and quantitative amounts of PUFA in the diet are crucial to the growth, survival and settlement of bivalve larvae. Next best growth rate and survival in *P. viridis* larvae observed in the present study when fed with *C. calcitrans* was also observed by AQUACOP (1979, 1983) when feeding *M. viridis* with mixture of *I. galbana* and *C. gracilas*. So a mixture of these two feeds either early or late in the culture period can enhance growth and settlement of *P. viridis* larvae.

Chlorella salina and *Nanochloropsis* sp. were found to be poor feeds for *P. viridis* larvae as was the case reported by several workers for *M. edulis* (Loosanoff and Davis, 1963; Bayne, 1965, 1983; Zong-Qing and Mei-Fang, 1980). According to Loosanoff and Davis (1963) the thickness in cell walls, production of toxic metabolites and non possession of enzymes to digest cellulose may be the reason for the non acceptance of these green microalgae as feed by the bivalve larvae. In addition, Tiu *et al.* (1989) found that total concentration of cellular protein within algal cells might be important in determining the quality of food. Low amount of protein in *Nanochloropsis* sp. may be responsible for its poor food acceptance or assimilation by *P. viridis* larvae.

Loosanoff and Davis (1963) in their exhaustive review on bivalve larval rearing reported that as far as qualitative food requirements are concerned, mussel *M. edulis* and clam *M. mercenaria* can ingest most microorganisms, provided they are also small enough to be ingested. Poor results observed with *Nanochloropsis* sp. which is smaller than the best feed *I. galbana* contradicts the above observation.

CHAPTER IV

FACTORS AFFECTING *PERNA VIRIDIS* LARVAL SETTLEMENT

1. INTRODUCTION

Aspects of settlement physiology of invertebrate larvae including bivalves have been reviewed by a number of workers (Crisp, 1974, 1976; Burke, 1983, 1986; Pawlik, 1992; Rodriguez *et al.*, 1993; Pechenik, 1999). It is now evident that many complex environmental factors contribute to the successful settling of bivalve larvae in the hatchery and as well as in the wild (Bonar *et al.*, 1990). The phenomenon of natural bivalve settlement involves a complex of behavioral, physiological and morphogenetic events. For the development of a viable bivalve hatchery technology, the identification of the conditions, which maximize larval settlement, is highly essential. In the present study the effects of physical factors like substratum, temperature, aeration, salinity, pH and chemical factors like L-DOPA, GABA, Serotonin, Potassium, Ammonia, Copper in the settlement of *Perna viridis* larvae were studied. In addition to this remote setting of the larvae was also observed.

To test the suitability of substratum, natural and artificial spat settling substrates have been used by various workers to collect mussel larvae in the wild and as well as in the hatchery (Bayne, 1976; Lutz and Kennish, 1992; Snodden and Roberts, 1997 ; Ramirez and Caceres-Martinez, 1999) .

In India studies on substrate preference of larvae have been carried out for *Mytilus viridis*, *Perna viridis* and *Perna indica* in controlled hatchery conditions, but detailed aspects of preferences have not been studied well (Rao *et al.*, 1976; Sivalingam, 1977; Rengarajan, 1983 *a*; Sreenivasan *et al.*, 1988 *a*; Appukuttan *et al.*, 1984, 1988).

There are numerous reports of preference of mussel larvae for filamentous surfaces like hydroids and various filamentous and coarsely branched algal species, in the field (Seed, 1976; Seed and Suchanek, 1992; Ostini *et al.*, 1994; Davis and Moreno, 1995; Moreno, 1995; Hunt and Scheibling, 1996) and in the hatchery (Bayne, 1964 *a*, 1965; Cooper, 1983; Petersen, 1984; Eyster and Pechenik, 1987; Newell *et al.*, 1991; Caceres-Martinez *et al.*, 1994).

Among the biological factors that influence the larval settlement, the effect of adults, the effect of sea weeds and the role of byssus threads have been studied in the present observations. Bayne (1976) had suggested a positive role for byssal threads in the primary settlement of mussel larvae. A number of workers have demonstrated the role of byssal filaments of adults on the settlement of mytilid larvae in the wild (Petraitis 1978; Suchanek, 1981; Moreno, 1995) and controlled conditions in the hatchery (Rengarajan, 1983 *a*; Trevelyan and Chang, 1983; Eyster and Pechenik, 1987; Caceres - Martinez *et al.*, 1994) . The role of adults in settlement of mussel larvae has also been reported both in the wild (Seed and Suchanek, 1992) and in the laboratory (Falmagne, 1983; Petersen, 1984; Caceres - Martinez *et al.*, 1994).

The effect of temperature on the settlement of mussel larvae has been studied by few workers (Bayne, 1965; Lutz *et al.*, 1970; Beaumont and Budd, 1982; Trevelyan and Chang, 1983; Taylor and Beattie, 1985; Pechenik *et al.*, 1990).

Bayne (1964 *a*, 1965) has investigated the effect of light on mussel larval settlement.

The role of aeration as a factor in the settlement of mussel larvae has also been studied by several workers (Siddall, 1979 *b*, 1980, 1982; Trevelyan and Chang, 1983; Taylor and Beattie, 1985; Eyster and Pechenik, 1987; Widdows, 1991; Lutz and Kennish, 1992).

Few studies have been reported on the effects of salinity on mussel larval settlement in the laboratory and in the wild, with reports of stimulating or no effect on settlement (Bayne, 1965; Siddall, 1979 *b*).

Bayne (1965) had indicated that pH could have some effect on the settlement of mussel larvae

Among the chemical factors affecting settlement of mussel *P. viridis*, L- DOPA, GABA, Serotonin, Ammonia, Pottasium and Copper have been studied.

L- DOPA (L - ß - 3, 4 - dihydroxy phenylalanine) a tyrosine derivative and a neurotransmitter precursor of dopamine is an active neuro- agent widely used to induce settlement of *Mytilus edulis*

(Cooper, 1982, 1983) and *M. galloprovincialis* (Satuito *et al.*, 1999) larvae.

Serotonin (5 - hydroxytryptamine) a derivative of tryptophan is a nueurotransmitter and a modulator of invertebrate nervous system. In *M. galloprovincialis,* Serotonin was found to be inductive only in high doses (Satuito *et al* ., 1999) .

GABA (γ – aminobutyric acid) was found to have no inductive effect in the blue mussel *M. edulis* (Cooper, 1982; Eyster and Pechenik, 1987) and the green mussel *Perna viridis* (Baylon, 1988).

Remote setting can be defined as a technique to get hatchery produced setting size pediveliger larvae shipped to near or distant locations for the final settlement as seeds on cultch material. Remote setting has revolutionized the bivalve aquaculture as oysters, clams, scallops or mussels can now be produced successfully by hatcheries at any time of the year and shipped as larvae to any corner of the world.

The advent of this technique has propelled the bivalve aquaculture industry forward in the sense that setting and nursery activities that once took place at a centralized hatchery are now dispersed about the country side and are conducted by individual growers rather than monopolized by few hatchery operators thus saving them time and money.

Remote setting has also been tried in mussels *M. edulis* (Trevelyan, 1989) and *M. galloprovincialis* (Kupier, 1991).

2. MATERIAL AND METHODS

All the experiments were conducted in shellfish hatchery at Tuticorin Research Centre of C.M.F.R.I, Tuticorin.

2.1. Conditioning of settlers

Settlers were washed thoroughly in clean tap water and then sterilized by autoclaving to remove all adhering debris and tissue materials. Materials were trimmed / cut to an uniform surface area of 5 cm^2 and washed in double distilled water. The materials were conditioned in fresh filtered running seawater in 100 l FRP tanks for 3 - 4 weeks. When a biofilm was formed, the substrate materials were removed from the tank, washed with sea water first and then with phosphate buffered saline (PBS pH = 7.4) to remove all debris and non adhered cells and introduced into settling tanks for substrate preference experiments. In all cases the substrate material was kept attached by plastic tape to the bottom of the tanks to prevent dislocation.

2.2. Experimental protocol

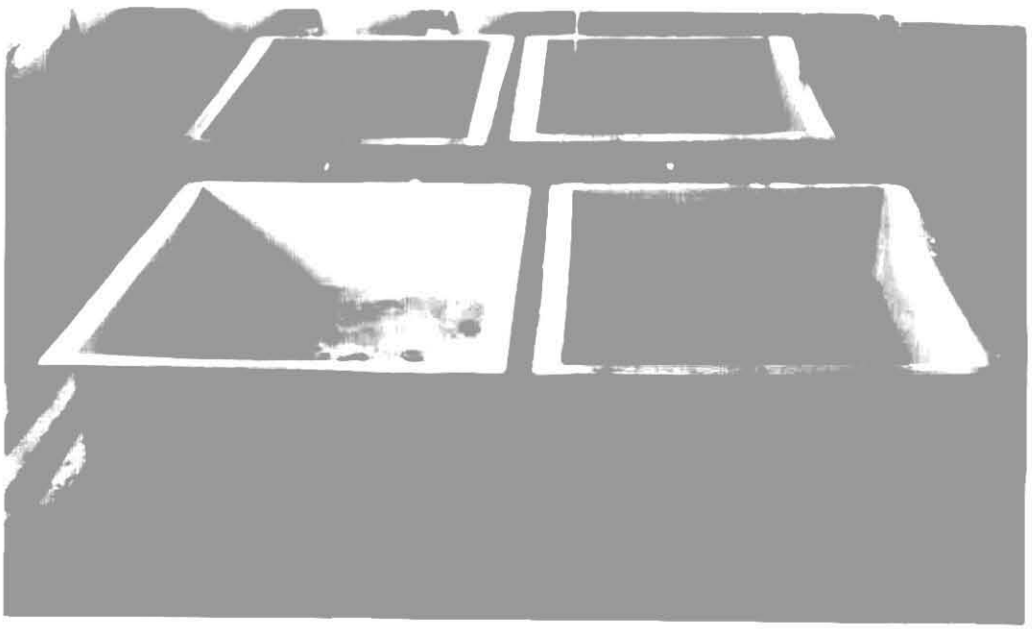
Larvae for the settlement experiments till pediveliger stage was reared (rearing protocols mentioned in the experimental protocol of Chapter III) in 1 ton FRP tanks (PLATE VI B). Hatchery produced healthy pediveliger larvae of green mussel *Perna viridis* with a well developed foot, swimming and crawling alternately and > 250 µm

shell length (270 ± 5.59 µm; (Antero – posterior length); 13 ± 2 day old) (PLATE V) were used for the experiments as per the classification of Bayne (1976) for all the primary settlement experiments.

For the physical (except for aeration experiments) and biological experiments settlement, the tanks / beakers/ tubs were mildly aerated and covered with a black cloth to prevent debris from falling into the tanks. The number of larvae attached to each substrate material after 24 hours (only for the adult and sea weed experiment) or 48 hours was noted (scored visually by observing attachment of the larvae by means of their byssal filaments) and converted into settlement percentage. If not specifically mentioned, all experiments were conducted in triplicates. Thousand pediveliger larvae were used for all the experiments except for the one on the effect of adults where five hundred mussel larvae each were added to every beaker.

For the experiments with chemical compounds, light and colour, the experimental assay was the same for all the compounds tested, with some minor modifications in time factor. Thirty pediveliger larvae were released gently into disposable sterilized plastic TARSONS petridishes containing 10 ml of the appropriate test solution prepared in fresh filtered sterilized (autoclaved) ambient sea water. The petridishes were closed and the experiment set up (PLATE VII B) was kept inside the hatchery where sufficient light falls on the petridishes. The number of larvae settled after 5, 12, 24 and 48 hours were scored visually (settlement of the larvae with the

PLATE VI

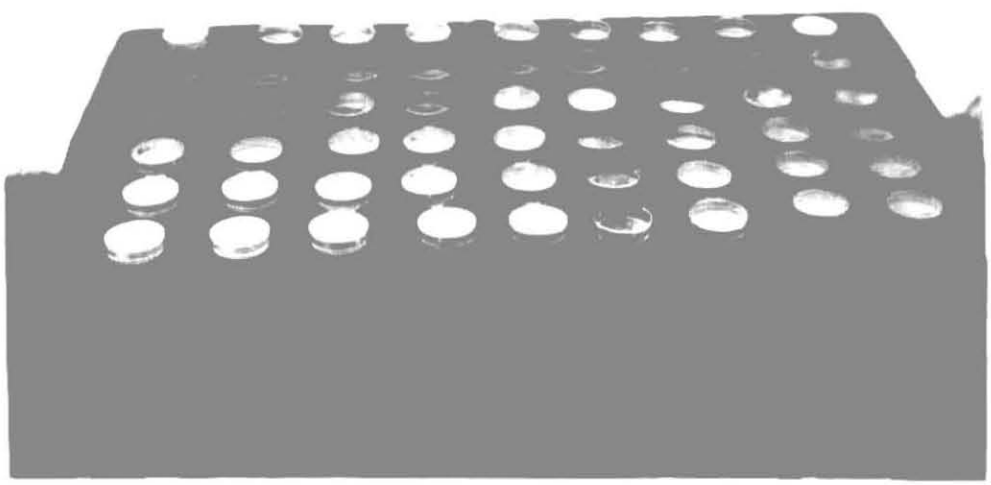


A. Larval rearing experiments in 100 l FRP tanks

B. Larval settlement experiments in 1 ton FRP tanks

PLATE VII

A. Controlled temperature experiments with Jumo thermometer and immersion heater



B. Settlement experiments with chemicals in petri dishes

combined effect of light, salinity and pH after 24, 48 hours and for colour alone after 48 hour was noted) and tabulated as percentage settled (mean ± standard deviation (S.D.)) at the end of the experiment. All the experiments were conducted in triplicates. For the experiments with chemicals, a control in ambient temperature, salinity and pH was maintained.

The effect of aeration, temperature, substrate preference, and effect of seaweeds was conducted in 100 l FRP tanks with 80 l fresh filtered sea water, whereas experiments on the effect of adults and remote setting were conducted in 5 l and 10 l glass beakers with 4 l and 8 l water respectively.

2.3. Physical and biological factors affecting settlement

2.3.1. Substrate preferences

For testing the primary substrate preferences for the primary settlement of *P. viridis* larvae, materials (settlers) like empty shells of mussel, clams and edible oyster, pieces of granite, laterite stone, coir rope, nylon monofilament, byssal threads, net pieces of nylon and velon screen were used as substrates.

2.3.2. Seaweeds

Seaweeds used as substrates were, *Ulva lactuca, U. reticulata, Chaetomorpha antennina, Enteromorpha compressa, Hypnea musciformis, Grateloupia filicina, Sargassum whightii* and *Padina gymnospora,* collected from Tuticorin and Mandapam coasts

in Tamil Nadu and from the harbour area in Pondicherry at low tide and transported to shellfish hatchery at Tuticorin. The cleaned, conditioned seaweeds were cut into pieces of 5 cm^2 (surface area) were used for the experiments. Similarly prepared monofilament and byssus threads, were used as controls for the experiment.

2.3.3. Presence of adult mussel

To find out the effect of adults on primary settlement of the green mussel *P. viridis*, pediveliger larvae of the species reared in the Tuticorin Shellfish hatchery of CMFRI were used. Adult and healthy mussels of 70 ± 5 mm (100 cm^2 surface area) were collected, washed, cleaned off foulers and other debris and introduced into 5 l tanks (one each). Strands of byssus threads and nylon monofilament (5 cm^2 surface area) were also introduced into every tank as control settlers in addition to the adult mussels (where settlement in 5 cm^2 surface area out of the total surface area was noted).

2.3.4. Temperature

Pediveliger larvae where made to settle at different sea water temperatures 31 °C, control (29 ° C), 27 ° C and 24 °C (controlled by a Jumo thermometer and immersion heater set up in an air conditioned room maintained at 23 ± 1 °C) (PLATE VII A).

2.3.5. Aeration

To find out the effects of aeration on *P. viridis* larval settlement, percentage settlement in moderate, heavy and no aeration (control), with and without settlers was studied. Settlers

used in each tank were three 5 cm ^{2} conditioned oyster shells arranged in a parallel row (PLATE VI A).

2.3.6. Light

The effect of continuous 300 - Lux bulb lighting on the settlement of mussel larvae was studied. The experiments were 1.fully covered with an aluminum foil to prevent the entry of light (dark) and 2. larvae in ambient light (control).

2.3.7. Colour

The effect of different colours on the settlement pattern of *P. viridis* was studied in ambient lighted conditions. The experiments were conducted under 1. The petridish totally covered with green, blue, black, red and yellow and white (control) colour 2. The bottom half of the petridish on the outside was covered by pasting green, blue, black, red, yellow and white (control) coloured papers.

2.4. Effect of chemicals on larval settlement

2.4.1. Salinity

Salinity 5 - 45 ppt. with control at 35 ppt was prepared by diluting fresh filtered sterilized sea water of ambient salinity 35 ppt. with filtered dechlorinised tap water for lower salinities and by adding freshly prepared sterilized brine to ambient salinity sea water. The experiments were conducted at 31 ° C.

2.4.2. pH

The effect of pH on larval settlement was tested in this experiment. For this experiment pH 4, 5, 6, 7, 8.2 (control) and 9 was prepared by adding 0.1 N HCl and 0.1 N NaCl to seawater of ambient pH 8.2. The experiments were conducted at 31 ° C

2.4.3. L - DOPA

L- DOPA (L- 3, 4 dihydroxy phenylalanine, Sigma) stock solutions of 1, 2.5, 3, 4, 5, 10 and 15 M concentrations were prepared in sterilized deionized glass distilled water to minimize the oxidation of the catechol moities which occur readily in sea water. (Coon *et al.*, 1985).

2.4.4. GABA

Gama - amino butyric acid (GABA) (Sigma) stock solutions of 1, 3, 4, 5, 10 and 15 M.

2.4.5. Serotonin

Serotonin (5 - Hydroxy Tryptamine, Sigma) stock solution of 1 and 3 M, were prepared in fresh filtered and sterilized sea water

2.4.6. Ammonia

2, 2.5, 3, 4, 4.5, 5, 10, 30 and 60 x 10^{-3} M concentration stock solution of Ammonia solution were made by dissolving analytical reagent grade Ammonium chloride (Qualigens, Glaxo) in

fresh filtered and sterilized sea water at twice the final concentrations mentioned above, and adjusted to pH 8.0 with NaOH.

2.4.7. Copper

Stock solutions of Copper chloride (Qualigens, Glaxo) at concentrations of 1.5, 2, 3, 4, 5, 6, 7, 8, 9 and 10 x 10^{-3} M were prepared just before the assay in fresh filtered sea water.

2.4.8. Pottasium

Stock solutions of Potassium chloride (Qualigens, Glaxo) at concentrations of 2, 4, 8, 12, 16, 20, and 30 x 10^{-3} M were prepared just before the assay in fresh filtered sea water

From the stock solutions concentrations ranging from 10^{-3} to 10^{-9} Molar (for L – DOPA, GABA and Serotonin) were prepared by serially diluting with filtered sterilized sea water just before the assay. The assay procedure was same as that mentioned earlier.

2.5. Water Quality parameters

Water quality parameters recorded during the different experimental trails of light, colour, pH, L – DOPA, GABA, Serotonin, Ammonia, Copper and Pottasium, were as follows. Air temperature: 37 ° C ambient and 23 ± 1 ° C in A.C.room, Water temperature: 31° C ambient and 23 ± 1 ° C in A.C. room, salinty 37 ±1 ppt, pH 8.2 ± 0.1, Dissolved Oxygen 5 ppm, Ammonia and H_2 S nil.

Water quality parameters for the remote setting experiment recorded at Tuticorin shellfish laboratory were the same as that in primary settlement experiment whereas in Cochin it was as follows, Air temperature: 34 ± 1° C, water temperature: 30 ± 1 ° C, salinty 34 ±1 ppt, pH 8.1 ± 0.1, Dissolved Oxygen, 4.6 ppm, Ammonia : 0.0015 ppm and H_2 S nil.

2.6. Remote setting

For remote setting experiments, pediveliger larvae of green mussel *Perna viridis* were used. Larvae were reared for 13 days at the Tuticorin shellfish hatchery of CMFRI until the pediveliger stage, as per protocols mentioned elsewhere in Chapter III on the larval rearing. Larval counts also were estimated as per previously mentioned protocols. The larvae were transported under three experimental conditions.

Thousand pediveliger larvae each were taken for each experimental unit in triplicates. The required larval suspension was filtered through a fine polyester cloth, folded and kept over a piece of cotton moistened with sea water and placed within a wide mouth transparent plastic bottle of 250 ml capacity. The mouth of the bottle was closed so that the larvae do not fall out during the transportation but allows sufficient air to pass inside. This is called moist packing at ambient temperature. Three other moist plastic bottle packages were transported under cool condition in an ice box maintained at 5 to 6 ° C below the ambient atmospheric temperature (25 ± 1 ° C) by putting crushed ice cubes in plastic covers. To maintain uniform

temperature and to avoid direct contact with the ice pieces, the chamber within the icebox was partitioned into an upper storage chamber and the lower ice chamber by means of a 2 cm thick perforated thermocool piece. Three moist packets of larvae were kept above this thermocool piece and the box was shut airtight. This is called moist packing at low temperature

Larvae were transferred into double layered polythene transportation bag filled with 1/3 rd (2 l of ambient sea water) and 2/3 rd with oxygen and packed tightly. These three oxygen filled packets containing larvae transported at ambient temperature (31 ± 1 ^{o} C) were treated as controls for the remote setting experiments. These packages of larvae were transported from Tuticorin shellfish laboratory of CMFRI to the hatchery complex of CMFRI at Kochi by road. The transportation time was 22 hours.

On arrival the larvae from each experimental unit was sampled for their survival during transport. The cloth containing larvae from each bottle was gently taken out and washed in slow running ambient filtered seawater for half an hour to acclimatize them to ambient rearing conditions. After estimation of the initial survival (after 24 hours) the larvae of each treatment was introduced into 5 l beakers filled with 4 l of ambient filtered sea water containing conditioned grits of oyster shells as substrates for settlement. The larvae were gently aerated during the experimental trial. The percentage survival and the number of larvae settled after 24 (initial) and final (48 hours post settlement) after the experiment started was noted individually by counting the attached spat on each settler.

2.7. Statistical analyses

Statistical analyses of the arcsine transformed settlement percentage data was analysed for primary substrate preferences, temperature, adults, sea weeds, different colours through a One – way Analysis of Variance (ANOVA) at $p < 0.05$ with EXCEL computer software.

In the case of aeration, a 2 – way ANOVA was conducted with aeration and substrate as source of variation. For the experiment on effect of light, a 2 – way ANOVA was conducted with time scales (24 and 48 hours), as the source of variation. For colour experiments too a 2 – way ANOVA was done between the different colours (48 hour data) for both totally covered and bottom coloured. This was followed by a One way ANOVA to find out the best colour for the settlement.

For salinity and pH settlement experiments a 2- way ANOVA was done between the 2 time scales (24 and 48 hours) and the salinity grades (15 - 45 ppt). A similar procedure was followed for pH. For experiments with chemicals like L – DOPA, a 2 – way ANOVA was done between 4 time scales (5, 12, 24 and 48 hours) and 8 concentrations of the test chemical dose (1, 2.5, 3, 4, 5, 10 and 15 M), followed by a 2 – way ANOVA between the sub concentration (10^{-3} to 10^{-9} M) and the 4 time scales (5, 12, 24 and 48 hours). Thereby the optimum level of the dosage and the appropriate time of administration were arrived. The same procedure was followed for Serotonin and GABA but with only 2 time scales 24 and 48 hours.

For ammonia and potassium, only a 2 – way ANOVA was conducted between 2 time scales (24 and 48 hours) and for copper (30 minutes and 1 hour) and the major concentrations to find out the optimum level of the dosage and the appropriate time of administration.

2 – way ANOVA using EXCEL computer software was conducted for remote setting experiments for the initial and final percentage survival and settlement.

In all the above experiments, if the F value of the treatments were significantly different then the best treatment was found out through pair wise (Students t – test ($p < 0.05$)) comparison of treatment means using Critical Difference (CD).

3. RESULTS

3.1. Physical and chemical factors

3.1.1. Substrate preference

Larvae of *P. viridis* preferred byssal threads most as substrate (37 ± 2.52 %) followed by nylon monofilament (14.33 ± 2.082 %) and then the control (12 ± 7 %). In velon screen, plastic rope and coir rope, the settlement was as low as 1.67 ± 0.57 %, 2 ± 0.71 % and 7.67 ± 1.41 % respectively, while 5.67 ± 0.71 % settled on the tank bottom (Fig. 27) (PLATE VIII A, B; IX A, B).

Statistically significant difference was observed in the percentage settlement of mussel larvae on the control and byssal threads. Significant difference also was noticed between byssus threads and nylon monofilaments. Byssal thread was the best substrate for green mussel larval settlement (Table 21).

3.1.2. Seaweeds

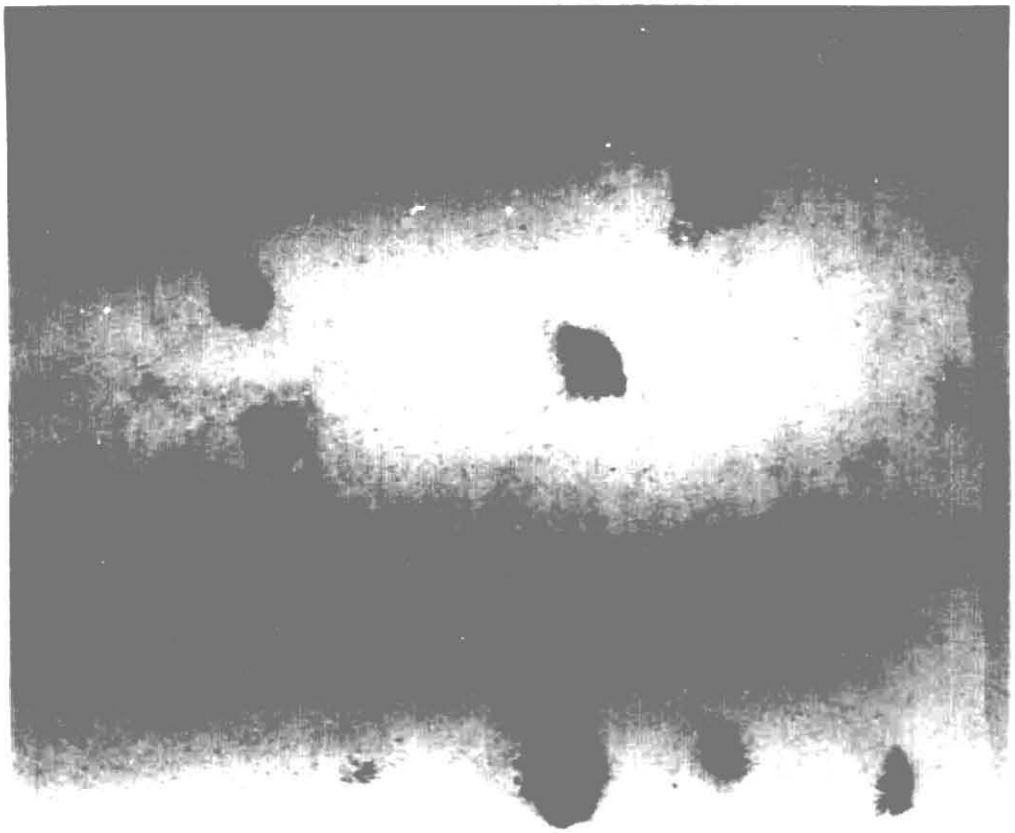
Among the seaweeds, maximum settlement was observed on *Chaetomorpha antenina* (4.33 ± 0.71 %). An almost uniform pattern of settlement was observed on all the other sea weeds *Enteromorpha compressa,* red algae *Grateloupia filicina*, *Hypnea musciformis* (about 1 - 1.5 %) when compared to settlement of 63.67 ± 5.033 % and 16 ± 1.41 % in byssal threads and nylon monofilaments respectively which were used as the controls (Fig.28).

PLATE VIII

A. Spat settled on filamentous substrate

B. Spat settled on hard substratum

PLATE IX



A. Two month old spat attached on smooth substratum

B. Two month old spat attached on hard substratum

No settlement was observed in brown algae. After statistical tests, among the seaweeds, *Chaetomorpha antennina* was found to be the best though very much inferior to the control (Table 22).

A simultaneous experiment to find out the settlement preference among different filamentous substrates revealed once more that byssus thread was the best among the filamentous substrates used for the primary larval settlement in *P. viridis.*

3.1.3. Presence of adult mussel

Results of the effect of adults on the primary larval settlement showed that 70.33 ± 6.028 % of the larvae attached to the adult shell surface while it was 17.33 ± 2.82 % and 7.67 ± 1.41 % for byssus threads and nylon monofilaments kept as control substrate materials after 24 hours (Fig. 29). Settlement was more on the ventral surface of shell valves of adults.

When statistically tested, it was seen that the percentage of larvae settling on adults was significantly better than byssal threads and nylon monofilaments (Table 23).

3.1.4. Temperature

The percentage settlement was 12 ± 4.36 %, 30.5 ± 4.27 %, 49.27 ± 4.34 % and 45.77 ± 6.87 % at 24 ° C, 27 ° C, control and 31 ° C respectively (Fig. 30). Settlement percentage did not differ significantly between 29 ° C (control) and 31 ° C but did so at 24 ° C

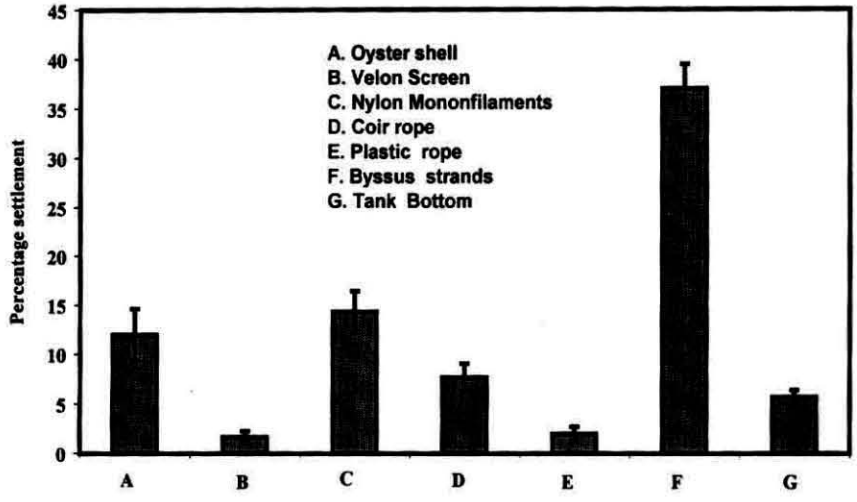


Fig. 27. Effect of different substrates on the settlement of *Perna viridis* larvae after 48 hours (vertical bars represent standard deviation)

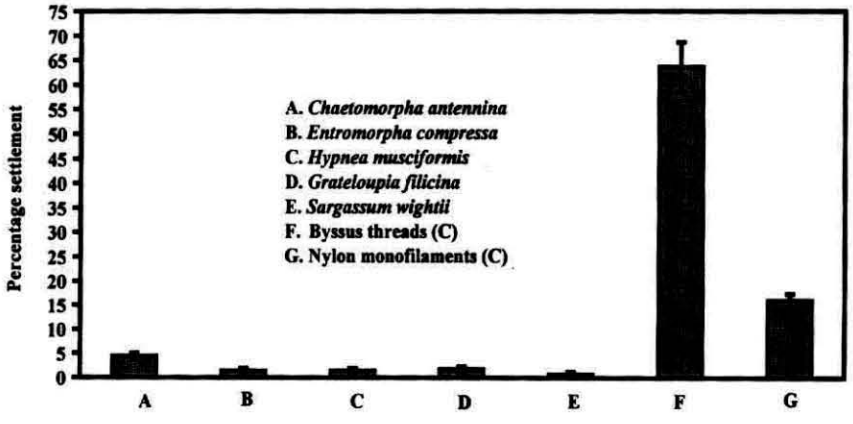


Fig. 28. Effect of seaweeds on *Perna viridis* larval settlement (vertical bars represent standard deviation)

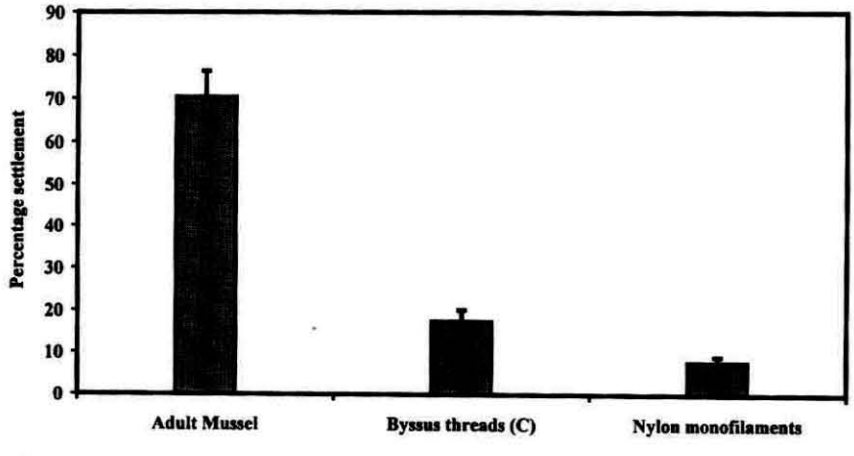


Fig. 29. Effect of adult mussels on *Perna viridis* larval settlement (vertical bars represent standard deviation)

and 27 ° C. Settlement percentage at 24 ° C and 27 ° differed significantly. A temperature range of 29 ° C – 31 ° C was best for the settlement (Table 24).

3.1.5. Aeration

The percentage settlement was 64.33 ± 4.04 %, 41.67 ± 2.89 %, 53 ± 2.65 % respectively in moderately, heavy and non aerated (control) in the presence of settlers (Fig. 31). On the other hand the percentage settlement was 32.33 ± 2.52 %, 12.33 ± 2.52 % and 22.33 ± 2.52 % respectively in moderately, heavy and non aerated (control) in the absence of settlers (Fig. 31).

When statistically tested all the treatments differed significantly. Statistical treatment of the results showed that irrespective of the treatment, percentage settlement in treatments with settlers was significantly better in ones with no settlers. The treatment of moderate aeration with a settlement substrate was the best for the settlement of *P. viridis* larvae than all the other treatments including the control (Table 25).

3.1.6. Light

P. viridis larval settlement in dark conditions (97.33 ± 2.31) was better after 24 hours compared to light (93.33 ± 8.33%) and control (83.33 ± 5.77%). After 48 hours the percentage settlement was nearly the same in lighted (98.33 ± 2.31 %), and dark conditions (97.33 ± 2.31 %). It improved marginally in the control

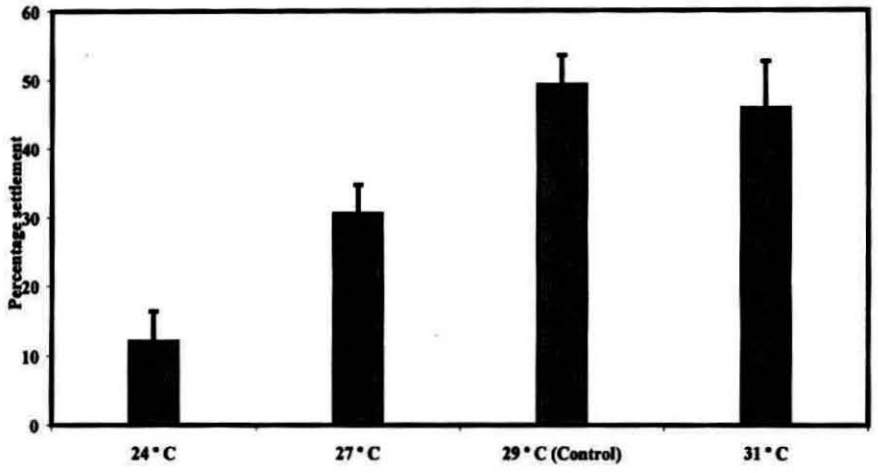


Fig. 30 . Effect of Temperature on *Perna viridis* larval settlement (vertical bars represent standard deviation)

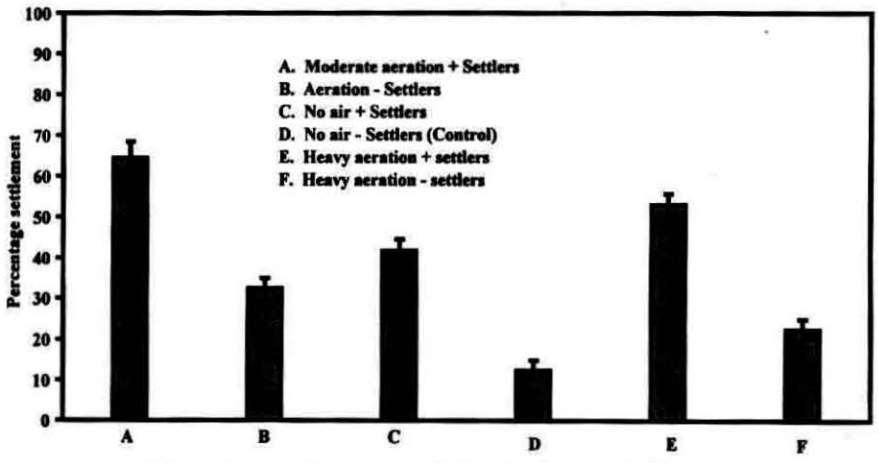


Fig. 31 . Effect of Aeration on *Perna viridis* larval settlement (vertical bars represent standard deviation)

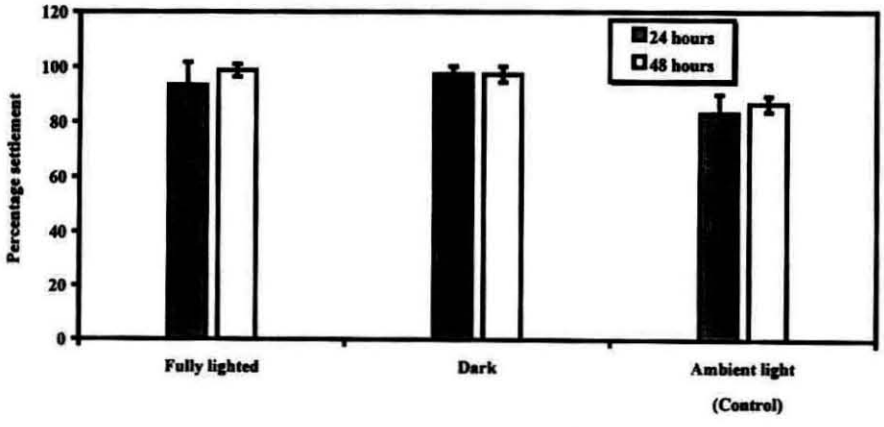


Fig. 32. Effect of Light on *Perna viridis* larval settlement (vertical bars represent standard deviation)

(86.67 ± 5.77 %) (Fig. 32). The treatments were not statistically significant (Table 26).

3.1.7. Colour

The preference of *P. viridis* larvae towards colours revealed that the percentage settlement was low in fully covered yellow, blue, green, red and black petridishes. At the end of 48 hours, the settlement percentages were 20 %, 33.33 ± 7.07 %, 36.67 ± 5.77 %, 43.33 ± 5.77 % , 70 ± 10 % in yellow, green, blue, red and black respectively, while 66.67 ± 5.77 % settled in the control (Fig. 33).

In the experiments with petridishes covered only on the top with the respective colours, the percentage settlement after 48 hours was 33.33 ±5.77 %, 36.67 ± 7.07 %, 40 ± 5.77 %, 46.67 ± 5.77 %, 63.33 ± 7.07 % respectively in yellow, green, blue, red and black at the same time 66.67 ± 5.77 % settled in control (Fig. 34).

Statistical analyses revealed that white (control) and black were significantly better for the settlement of *P. viridis* larvae when compared to other colours which though did not differ significantly among themselves (Table 27).

3.2. Effect of chemicals on larval settlement

3.2.1. Salinity

In the salinity tolerance experiments, all the larvae were dead at 5 and 10 ppt within two hours in both the temperatures tested. No

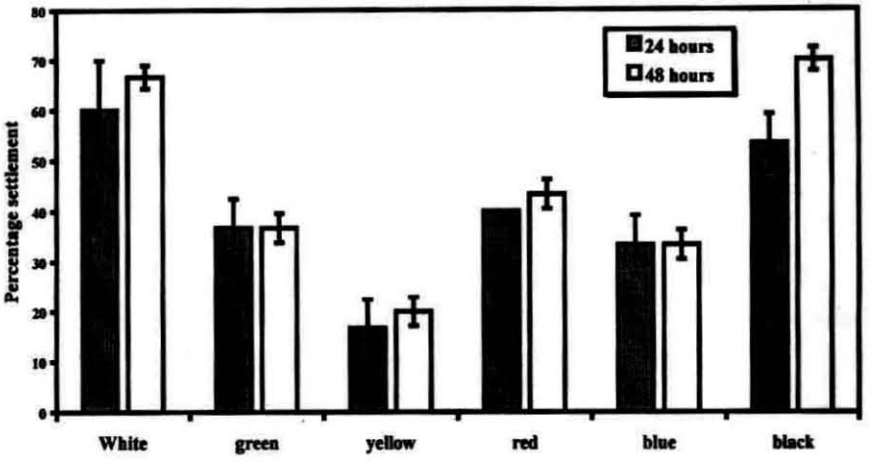


Fig. 33 . Effect of total colour on *Perna viridis* larval settlement (vertical bars represent standard deviation)

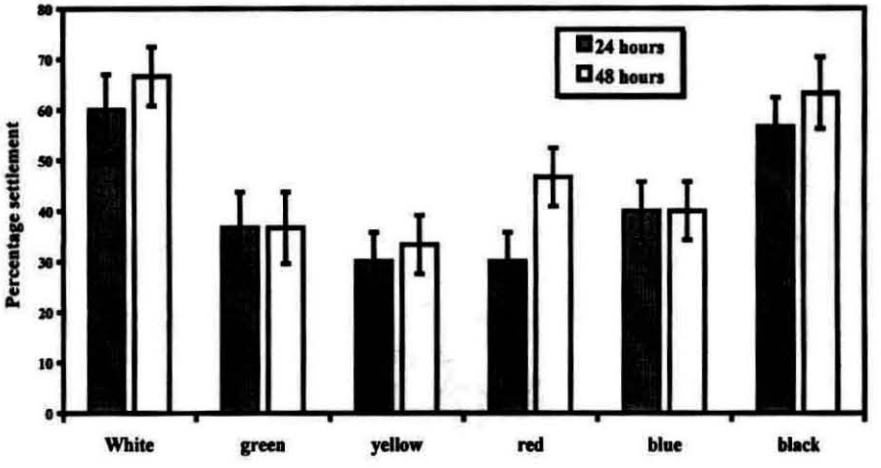


Fig. 34. Effect of bottom colour on *Perna viridis* larval settlement (vertical bars represent standard deviation)

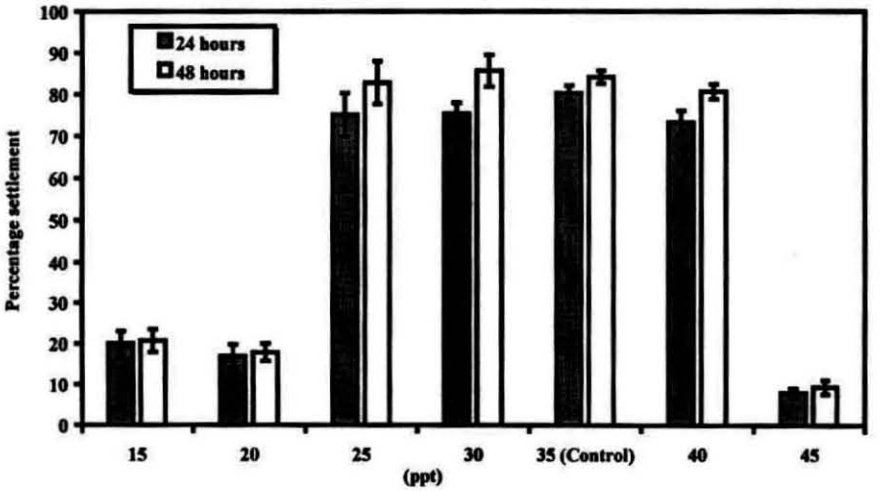


Fig. 35. Effect of salinity on *Perna viridis* larval settlement (vertical bars represent standard deviation)

settlement was observed in any of the salinity treatments till 24 hours. The percentage settlement after 24 hours at different salinities were 20 ± 3.027 %, 16.88 ± 2.86 %, 75.1 ± 5.25 %, 75.31 ± 2.64 %, 80.31 ± 1.86 %, 73.25 ± 2.81 %, 7.9 ± 1.16 % for 15, 20, 25, 30, 35 (Control), 40, 45 ppt respectively (Fig. 35). At 48 hours the percentage settlement was 20.7 ± 2.81 %, 17.87 ± 2.15 %, 82.83 ± 5.15 %, 85.7 ± 3.86 %, 84.2 ± 1.53 %, 80.75 ± 1.77 %, 9.34 ± 1.71 % for 15, 20, 25, 30, 35 (Control), 40, 45 ppt respectively (Fig. 35).

Statistical analysis revealed that there was significant difference between the percentage settlement at different salinities. Salinity range 25 - 40 ppt at 48 hours was the best for the settlement of *P. viridis* larvae (Table 28).

3.2.2. pH

In pH tolerance experiments even though larvae settled at extreme pH range there was considerable mortality. The percentage settlement of larvae after 24 hours was 23.067 ± 3.002 %, 19.2 ± 2.27 %, 45.57 ± 1.9.4 % , 39.1 ± 5.57 % and 30.11 ± 3.167 % in pH 5, 6, 7, control and pH 9 respectively (Fig. 36). Forty eight hour settlement pattern showed 40.92 ± 3.58 %, 50.2 ± 4.91 %, 70.52 ± 2.19 %, 75.33 ± 5.69 % and 50.40 ± 3.62 % settlement at pH 5, 6, 7, control and pH 9 (Fig. 36).

Statistical analysis of pH tolerances clearly showed that pH influenced significantly in the settlement of *P. viridis* larvae. The

percentage settlement of *P. viridis* larvae was best in the pH range of 7 – 8 after 48 hours (Table 29).

3.2.3. L - DOPA

Settlement pattern was studied for the effect of L – DOPA at different test doses and at different times and the percentage settlement is given in Table 18. L- DOPA at 2.5×10^{-7} M , nearly 100 % settlement was observed within 5 hours (96.77 ± 2.12 %) (Table 30). Statistical treatment indicated that the optimum concentration of L - DOPA for settlement of *P. viridis* larvae was 2.5×10^{-7} M at 5 hours (Table 30).

3.2.4. GABA

For GABA the percentage of settlement at different time intervals at different test concentrations were recorded in Table 19. GABA showed no specific inductive effect except in very high concentrations 10 and 15×10^{-3} to 10^{-9} M levels. The best percentage of settlement was observed in ranges from 10^{-4} to 10^{-6} M in 24 and 48 hours (Table 31). The percentage settlement decreased drastically as the dilution increased.

Statistical analysis of the percentage settlement data indicated that the optimum concentration of GABA for settlement of *P. viridis* larvae was best at $10 – 15 \times 10^{-6}$ M at 24 – 48 hours (range $1 – 15 \times 10^{-3}$ - 10^{-9} M) even though 15×10^{-6} M at 24 – 48 hours was marginally better (Table 31).

Table 18. Effect of L-DOPA on the settlement of *Perna viridis* larvae
Percentage settlement (Mean ± S. D.)

Concentration (M)	Time (Hrs)	Mean ± S.D							
		10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	Control
1	5	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	12	2.38 ± 0.13	4.17 ± 0.22	13.33 ± 0.77	6.25 ± 0.00	4.71 ± 0.83	2.22 ± 0.39	4.17 ± 0.72	0.00 ± 0.00
	24	16.68 ± 1.83	8.33 ± 1.44	26.67 ± 0.58	16.67 ± 1.72	14.30 ± 2.48	6.67 ± 1.15	8.33 ± 1.44	10.40 ± 2.86
	48	45.27 ± 1.10	29.17 ±2.22	26.67 ± 1.15	22.92 ± 1.30	54.20 ± 1.43	35.58 ± 2.85	41.67 ± 1.91	24.20 ± 2.63
2.5	5	8.33 ± 0.43	50.00 ± 0.00	00.00 ± 0.00	47.67 ± 1.65	98.77 ± 2.12	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	12	16.67 ± 2.79	50.00 ± 0.00	5.56 ± 1.62	52.43 ± 2.18	100.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	24	58.33 ± 3.82	66.67 ± 2.89	22.22 ± 2.62	66.73 ± 2.18	100.00 ± 0.00	28.23 ± 1.18	8.33 ± 0.00	10.40 ± 2.86
	48	66.67 ± 2.89	66.67 ± 2.89	47.22 ± 1.74	90.50 ± 1.65	100.00 ± 0.00	64.17 ± 1.18	11.11 ± 1.82	24.20 ± 2.53
3	5	22.22 ± 1.92	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	12	33.33 ± 1.67	6.67 ± 1.15	6.67 ± 1.15	25.00 ± 0.00	11.11 ± 1.92	0.00 ± 0.00	8.33 ± 1.44	0.00 ± 0.00
	24	55.56 ± 2.62	20.00 ± 0.00	46.67 ± 3.55	58.33 ± 1.43	38.89 ± 1.62	14.28 ± 0.00	25.00 ± 0.00	10.40 ± 2.86
	48	77.78 ± 2.24	80.00 ± 0.00	80.00 ± 6.00	83.33 ± 2.43	83.32 ± 1.67	71.40 ± 1.43	75.00 ± 2.50	24.20 ± 2.53
4	5	27.78 ± 2.62	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	12	33.33 ± 0.00	0.00 ± 0.00	2.78 ± 0.81	2.08 ± 0.61	4.76 ± 0.24	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	24	55.56 ± 3.62	16.67 ± 0.00	16.66 ± 1.44	8.53 ± 1.61	23.80 ± 1.65	8.89 ± 1.08	16.67 ± 0.00	10.40 ± 2.86
	48	66.67 ± 0.00	38.89 ± 2.62	57.33 ± 1.52	16.67 ± 2.22	61.88 ± 1.24	66.63 ± 1.77	30.55 ± 2.81	24.20 ± 2.53
5	5	4.34 ± 0.00	23.33 ± 1.15	9.53 ± 2.26	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	12	4.34 ± 0.00	26.67 ± 1.77	9.53 ± 1.26	3.74 ± 1.47	4.17 ± 1.22	5.56 ± 0.96	11.11 ± 1.92	0.00 ± 0.00
	24	4.34 ± 0.00	33.00 ± 1.20	28.60 ± 1.43	22.29 ± 1.92	12.50 ± 2.17	16.67 ± 2.89	16.67 ± 1.89	10.40 ± 2.86
	48	11.57 ± 0.01	40.00 ± 0.00	42.90 ± 1.48	29.63 ± 3.21	33.34 ± 2.89	33.34 ± 1.89	22.22 ± 3.85	24.20 ± 2.53
10	5	6.67 ± 0.77	8.33 ± 1.44	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	12	13.33 ± 0.77	16.67 ± 2.89	4.17 ± 2.22	4.17 ± 1.22	8.33 ± 1.44	2.57 ± 2.45	8.33 ± 1.44	0.00 ± 0.00
	24	30.00 ± 1.73	100.00 ± 0.00	25.00 ± 2.17	29.17 ± 1.09	25.00 ± 0.00	17.97 ± 1.78	25.00 ± 0.00	10.40 ± 2.86
	48	46.67 ± 2.08	100.00 ± 0.00	75.00 ± 2.50	50.00 ± 2.17	66.67 ± 1.18	56.47 ± 1.18	83.33 ± 2.43	24.20 ± 2.53
15	5	1.67 ± 2.89	19.06 ± 1.26	0.00 ± 0.00	0.00 ± 0.00	5.00 ± 2.66	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	12	10.00 ± 5.00	33.37 ± 2.26	2.22 ± 0.85	2.67 ± 1.62	8.33 ± 1.44	6.67 ± 1.15	0.33 ± 0.58	0.00 ± 0.00
	24	33.33 ± 1.04	52.43 ± 1.51	17.79 ± 1.77	10.67 ± 2.62	100.00 ± 0.00	20.00 ± 1.02	1.00 ± 0.00	10.40 ± 2.86
	48	88.33 ± 1.26	100.00 ± 0.00	93.33 ± 1.15	28.00 ± 1.01	100.00 ± 0.00	31.12 ± 1.08	3.00 ± 1.40	26.20 ± 2.53

Table 19. Effect of GABA on the settlement of *Perna viridis* larvae
Percentage settlement (Mean ± S. D.)

Concentration (M)	Time (Hrs)	Mean ± S.D							
		10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	Control
1 M	5	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	12	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	24	26.67 ± 1.15	22.21 ± 1.92	40.74 ± 2.41	45.83 ± 2.22	4.04 ± 0.75	10.00 ± 0.00	3.70 ± 2.41	10.40 ± 2.86
	48	40.00 ± 1.00	33.33 ± 1.92	55.55 ± 1.11	58.33 ± 1.22	9.09 ± 6.25	10.00 ± 0.00	3.70 ± 1.41	24.20 ± 2.53
3 M	5	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	12	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	24	9.52 ± 1.24	9.52 ± 2.24	37.50 ± 0.00	40.00 ± 0.00	5.53 ± 0.40	1.92 ± 0.00	3.47 ± 0.50	10.40 ± 2.86
	48	16.36 ± 1.86	38.40 ± 1.91	45.83 ± 1.44	60.00 ± 0.00	12.48 ± 0.00	12.18 ± 1.43	5.89 ± 1.00	24.20 ± 2.53
4 M	5	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	12	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	24	45.83 ± 1.22	33.33 ± 0.00	18.33 3.18	16.67 ± 0.00	33.33 ± 2.31	26.671 ± 1.16	25.00 ± 0.00	10.40 ± 2.86
	48	58.33 ± 2.22	33.33 ± 0.00	50.00 ± 2.66	53.33 ± 2.31	50.00 ± 1.67	33.33 ± 0.00	33.33 ± 0.00	24.20 ± 2.63
5 M	5	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	12	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	24	25.00 ± 0.00	57.37 ± 1.43	11.11 ± 1.92	33.33 ± 0.00	10.53 ± 1.81	10.52 ± 1.11	3.22 ± 0.00	10.40 ± 2.88
	48	25.00 ± 0.00	71.50 ± 0.00	33.33 ± 0.00	83.33 ± 1.22	26.30 ± 1.82	22.79 ± 1.04	9.66 ± 1.58	24.20 ± 2.53
10 M	5	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	12	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	24	45.83 ± 1.44	12.50 ± 0.00	33.33 ± 1.43	50.00 ± 0.00	50.00 ± 0.00	28.23 ± 2.45	12.50 ± 0.00	10.40 ± 2.86
	48	62.50 ± 0.00	33.33 ± 2.43	75.00 ± 2.50	80.00 ± 0.00	58.33 ± 1.44	35.93 ± 1.89	12.50 ± 0.00	24.20 ± 2.53
15 M	5	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	12	22.22 ± 0.00	20.00 ± 0.00	28.67 ± 1.15	25.00 ± 0.00	12.50 ± 2.17	8.33 ± 1.44	0.00 ± 0.00	0.00 ± 0.00
	24	44.44 ± 1.11	43.33 ± 5.77	53.33 ± 2.31	80.00 ± 0.00	37.50 ± 0.00	25.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	48	70.36 ± 2.41	56.67 ± 1.77	66.67 ± 1.15	93.33 ± 1.55	50.00 ± 1.65	33.33 ± 2.43	25.00 ± 0.00	24.20 ± 2.53

3.2.5. Serotonin

Settlement pattern was studied for the effect of Serotonin at different test doses and at different times and the percentage settlement is given in Table 20. Settlement was high in the highest concentrations and decreased to a very low value at the lowest concentration, but not showing any specific pattern. In all concentrations from 1×10^{-5} to 10^{-9} M Serotonin, there was no settlement upto 24 hours. 3 M concentration was found to be inhibitory factor for settlement of *P. viridis* larvae as percentage settlement in this concentration was only half that 1×10^{-3} to 10^{-9} M concentrations.

After statistical treatment, the optimum concentration of Serotonin for settlement of *P.viridis* larvae was found to be best at 1×10^{-6} - 10^{-8} M at 48 hours (Table 32).

3.2.6. Ammonia

Ammonia failed to elicit any settlement response till 24 hours after administration. The general trend indicated that settlement increased with increased concentration upto 30 mM then decreased at very high concentration of 60 mM (Fig. 37). After 24 hours, best settlement of 62 ± 3.89 % occurred in 10 mM. 7.67 ± 0.71 %, 8.33 ± 1.53 %, 14.75 ± 0.71 %, 14.66 ± 1.15 % 37.83 ± 3.75 %, 41.29 ± 0.37 %, 61.353 ± 3.67 % and 30 % settlement was observed in 2 mM, 2.5mM, 3mM, 4mM, 4.5 mM, 5 mM, 30 mM and 60 mM respectively. In controls 11.69 ± 2.98 % numbers settled. (Fig. 37).

Table 20. Effect of Serotonin on the settlement of *Perna viridis* larvae
Percentage settlement (Mean ± S. D.)

Concentration (M)	Time (Hrs)	Mean ± S.D							
		10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	Control
1 M	5	56.39 ± 7.5	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	12	72.51 ± 7.6	11.18 ± 2.68	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	24	72.71 ± 7.9	41.11 ± 4.56	12.43 ± 2.75	27.88 ± 5.82	58.58 ± 6.77	63.43± 5.49	55.9 ± 5.78	10.4 ± 2.88
	48	94.34 ± 7.79	95.96 ± 5.46	83.86 ± 4.71	93.37 ± 3.79	95.86 ± 5.03	86.57 ± 5.56	85.55 ± 6.54	24.20 ± 2.53
3 M	5	34.76 ± 6.39	23.36 ± 9.8	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	12	35.28 ± 6.32	23.01 ± 2.94	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	24	68.89 ± 6.73	45.76 ± 7.98	20.33 ± 3.51	49.1 ± 0.07	37.26 ± 6.27	32.33 ± 4.95	49.98 ± 0.71	10.40 ± 2.88
	48	92.69 ± 7.19	95.90 ± 4.36	40.00 ± 5.60	21.97 ± 3.05	48.64 ± 6.13	49.98 ± 6.74	21.97 ± 3.05	24.20 ± 2.53

At 48 hours, percentage settlement was very low with 12.81 ± 0.44 % in 2 mM which increased to a best of 97.19 ± 0.51 % in 10 mM compared to 24 ± 3.11 % in controls. 32 ± 2.89 %, 33.2 ± 3.3 %, 37.23 ± 3.70 % 77.48 ± 2.26 %, 81.64 ± 4.06 %, 75.56 ± 2.35 % and 74.7 ± 3.38 % settlement was observed in 2.5mM, 3mM, 4mM, 4.5 mM, 5 mM, 30 mM and 60 mM respectively (Fig. 37).

Ammonia at 10 mM at 48 hours was found to be the best for the settlement of *P. viridis* larvae when statistically analysed (Table 33).

3.2.7. Copper

In Copper, nearly 80 % (a low of 82.94 ± 5.29 % in 8 mM, 85.33 ± 0.58 % in 6 mM, 85.39 ± 4.65 % in 5 mM, 86.14 ± 3.99 % in 4 mM, 88.22 ± 7.45 % in 10 mM, 88.67 ± 7.1 % in 7 and 9 mM respectively 98 ± 2 % in 2 mM and 100 % in 3 mM) of *P. viridis* larvae exposed to millimolar concentrations of copper settled in less than half an hour and all after 1 hour of exposure, while none settled in the control (Fig. 38). Larvae exposed to copper showed heavy mortality after 5 hours of continuous exposure in all the concentrations tested. 2 – 3 mM in 30 minutes was the best treatment for larval settlement of *P. viridis* (Table 34).

3.2.8. Potassium

In Potassium, the percentage settlement increased with increase in concentration upto 30 mM and then decreased in higher

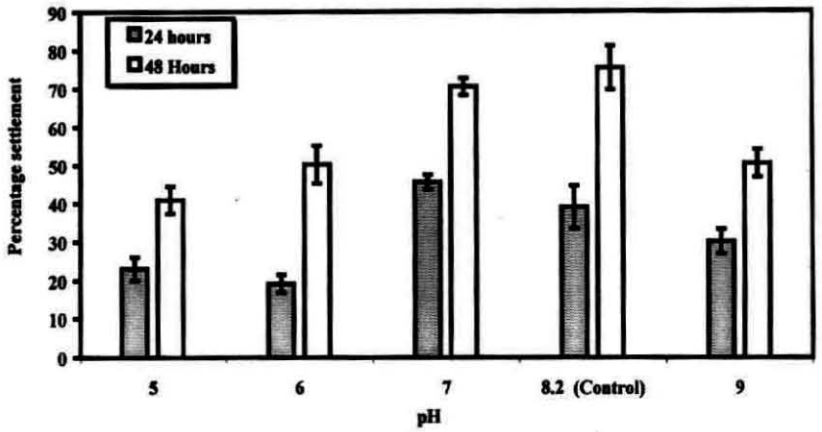


Fig. 36. Effect of pH on *Perna viridis* larval settlement (vertical bars represent standard deviation)

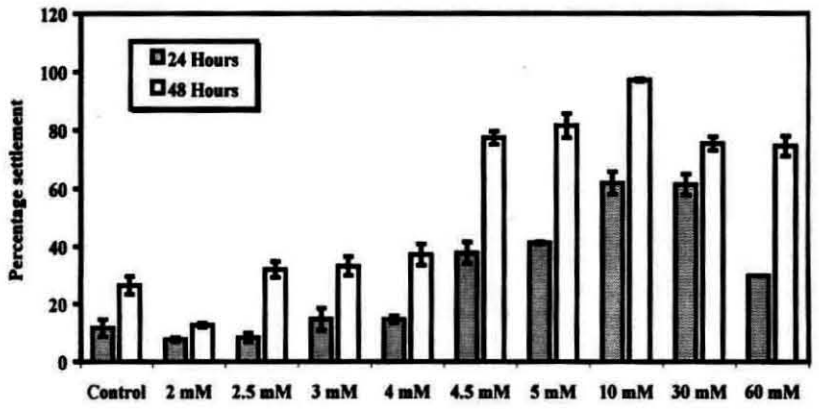


Fig. 37. Effect of Ammonia on *Perna viridis* larval settlement (vertical bars represent standard deviation)

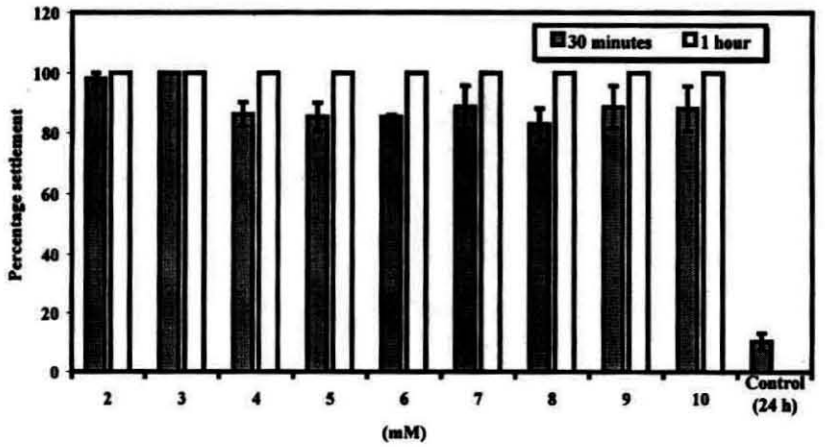


Fig. 38. Effect of Copper on *Perna viridis* larval settlement (vertical bars represent standard deviation)

concentrations of 40 and 60 mM irrespective of the time. None of the *P.viridis* larvae settled after 5 hours of exposure. At the end of 12 hours none settled in 2 and 4 mM. The percentage settlement was 19.67 ± 3.54 %, 24.67 ± 3.54 %, 33.86 ± 3.41 %, 34.4 ± 1.65 %, 50.33 ± 7.75 %, 24.7 ± 2.52 % and 25.33 ± 1.41 % for 5, 8,1 2, 20, 30, 40 and 60 mM concentrations respectively (Fig. 39). After 24 hours there was only a marginal rise in the settlement percentage in all the treatments. (11.42 ± 1.8 %, 20.33 ± 0.71 %, 21 ± 1.41 %, 25.33 ± 2.83 %, 34.91 ± 1.87 %, 35.6 ± 0.33 %, 52.33 ± 4.95 %, 27.67 ± 1.43 % and 29.5 ± 4.94 % in 2,4, 5, 8, 12, 20, 30, 40 and 60 mM respectively. In the control, 10.3 ± 2.86 % settled in the same time period (Fig. 39). After 48 hours there was a marginal rise in the settlement in 4, 5, 12, 20, 30 mM and percentage settlement being 25.3 ± 1.53 %, 26 ± 1.41 %, 35.7 ± 2.81 %, 37 ± 1.18 %, 53.7 ± 4.24 %) respectively. There was no change in 8 mM, 40 mM and 60 mM. In control and 2mM there was a 2 - fold increase in the settlement percentage when compared to 24 hour data (24.2 ± 2.53 % and 21.7 ± 2.77 % respectively) (Fig. 39).

Statistical analyses revealed that potassium at 30 mM at 48 hours was the best for the settlement of *P. viridis* larvae (Table 35).

3.3. Remote setting

Percentage survival after transportation was 82.67 ± 4.62 %, 74.33 ± 3.79 % and 65.67 ± 5.13 % in control, moist transported larvae at ambient temperature and moist transported larvae at low temperature respectively (Fig. 40). At the end of 24 hours post

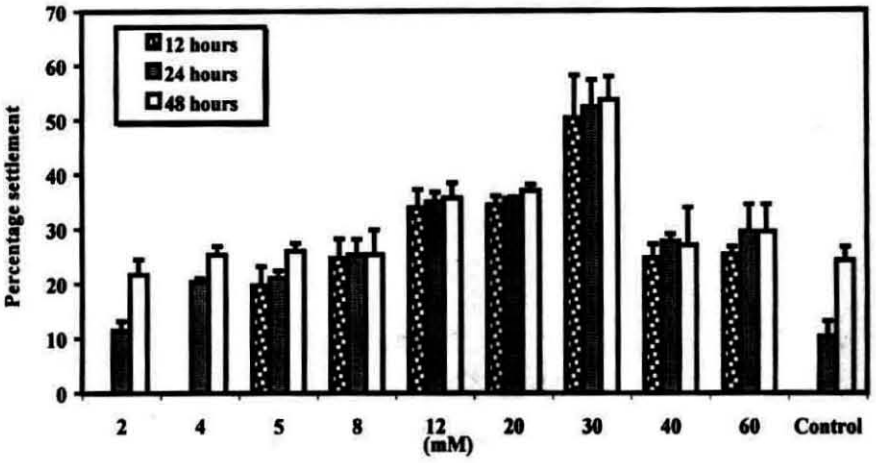


Fig. 39. Effect of Potassium on the settlement of *Perna viridis* larvae (vertical barsrepresent standard deviation)

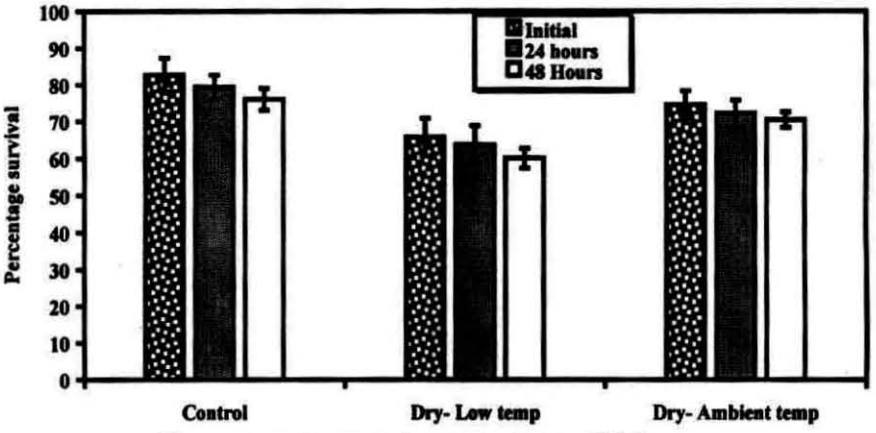


Fig. 40. Percentage survival in remotely set *Perna viridis* larvae (vertical barsrepresent standard deviation)

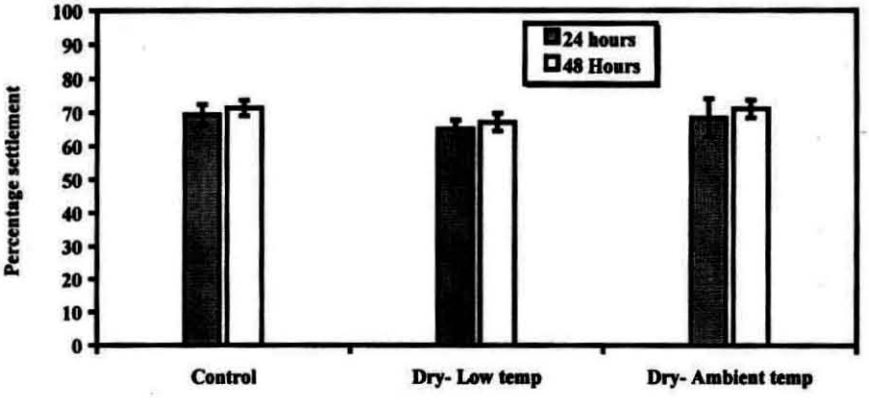


Fig. 41. Percentage settlement in remotely set *Perna viridis* larvae (vertical bars represent standard deviation)

settlement (48 hours after transport), the percentage survival was 79.33 ± 3.21 % in control, 72 ± 3.61 % for moist transported larvae at ambient temperature and 63.67 ± 5.13 % for moist transported at low temperature. Data on the survival rate at the end of 48 hours (96 hours after transport) showed that survival was only marginally different in moist transported larvae at ambient temperature (70.33 ± 2.08 %) when compared with the control (76 ± 3 %), but moist transported larvae at low temperature showed a reduction in survival (60 ± 2.65 %) (Fig. 40).

As far as the settlement rate was concerned there was little to compare between the control and moist transported larvae at ambient temperature while moist transported larvae at low temperature showed less settlement rates. After 24 hours the percentage of settlement was 69.33 ± 3.06 %, 68.53 ± 5.69 % and 65 ± 2.65 % for control, moist transported at ambient temperature and moist transported at low temperature respectively. After 48 hours post settlement (72 hours after transport), the percentage settlement increased only slightly among all the treatments. It was 71.33 ± 2.31 %, 71 ± 5.69 % and 67 ± 2.65 % for control, moist transported without water at ambient temperature and moist transported without water at low temperature (Fig. 41).

Test of significance for survival revealed no significant difference in the percentage survival between the time intervals - initial, 24 and 48 hours. Survival percentage of the moist transported at low temperature was significantly low compared to that of the

control while no significant difference was observed between the control and larvae transported moist at ambient temperature (Table 36).

Treatments did not differ statistically from the control when the percentage settlement data were analyzed, both at 24 or 48 hours post settlement. There was also no statistically significant difference between the percentage of larvae settling in any treatment between 24 or 48 hour post settlement (Table 36).

Table 21. Effect of substrates on *P.viridis* larval settlement

One Way Analysis of Variance (ANOVA)

Source of Variation	SS	df	MS	F	P-value
Substrates	2682.57	6.00	447.10	45.14	0.00 *
Within	138.67	14.00	9.90		

Table 22. Effect of seaweeds on *P.viridis* larval settlement

One Way Analysis of Variance (ANOVA)

Source of Variation	SS	df	MS	F	P-value
Seaweeds	5519.40	6.00	919.90	130.98	0.00 *
Within	98.33	14.00	7.02		

Table 23. Effect of adult mussel on *P.viridis* larval settlement

One Way Analysis of Variance (ANOVA)

Source of Variation	SS	df	MS	F	P-value
Adult	2867.20	2.00	1433.60	5.14	0.00 *
Within	113.08	6.00	18.85		

Table 24. Effect of temperature on *P.viridis* larval settlement

One Way Analysis of Variance (ANOVA)

Source of Variation	SS	df	MS	F	P-value
Temperature	1124.02	3.00	374.67	13.22	0.00 *
Within	226.72	8.00	28.34		

Table 25. Effect of aeration on *P.viridis* larval settlement

Two way Analysis of Variance (2 - Way ANOVA)

Source of Variation	SS	df	MS	F	P-value
Aeration	1365.30	2.00	682.87	80.84	0.00 *
Settlers	4232.00	1.00	4232.00	501.16	0.00 *
Interaction	5.33	2.00	2.67	0.32	0.74
Within	10.33	12.00	8.44		

Table 26. Effect of light on *P.viridis* larval settlement

Two way Analysis of Variance (2 - Way ANOVA)

Source of Variation	SS	df	MS	F	P-value
Light	785.84	2.00	392.92	9.15	0.10
Time (24, 48 Hours)	49.63	1.00	49.63	1.16	0.30
Interaction	40.25	2.00	20.13	0.47	0.64
Within	515.58	12.00	42.97		

Table 27. Effect of colour on *P.viridis* larval settlement

Two way Analysis of Variance (2 - Way ANOVA)

Source of Variation	SS	df	MS	F	P-value
Top and bottom colour	89.97	1.00	89.97	3.11	0.90
Between colours	1025.76	5.00	205.15	7.10	0.00 *
Interaction	98.67	5.00	179.74	6.22	0.00 *
Within	693.65	24.00	28.90		

Table 28. Effect of salinity on *P.viridis* larval settlement

Two way Analysis of Variance (2 - Way ANOVA)

Source of Variation	SS	df	MS	F	P-value
Salinity 15 - 45 ppt	14781.26	6.00	2463.54	67.41	0.00 *
Time (24, 48 Hours)	173.77	1.00	173.77	4.76	0.38
Interaction	157.43	6.00	26.24	0.72	0.64
Within	1023.27	28.00	36.55		

Table 29. Effect of pH on *P.viridis* larval settlement

Two way Analysis of Variance (2 - Way ANOVA)

Source of Variation	SS	df	MS	F	P-value
pH 5 - 9	1385.14	4.00	346.28	58.99	0.00 *
Time (24, 48 Hours)	1884.01	1.00	1884.01	320.95	0.00 *
Interaction	138.42	4.00	34.60	5.90	0.03 *
Within	117.40	20.00	5.87		

Table 30. Effect of L - DOPA on *P.viridis* larval settlement

Two way Analysis of Variance (2 - Way ANOVA)

Source of Variation	SS	df	MS	F	P-value
Between 1 M and 15 M	29120.65	6.00	4853.44	13.80	0.00 *
Time (5, 12 24, 48 Hours)	161923.58	3.00	53974.53	153.50	0.00 *
Interaction	15203.77	18.00	844.65	2.40	0.00 *
Within	226442.04	644.00	351.62		
Between 2.5 x 10^{-3} and 10^{-9} M	64933.79	7.00	9276.26	59.23	0.00 *
Time (5, 12 24, 48 Hours)	14636.97	3.00	4878.99	31.16	0.00 *
Interaction	6062.98	21.00	288.71	1.84	0.03 *
Within	10022.79	64.00	156.61		

Table 31. Effect of GABA on *P.viridis* larval settlement

Two way Analysis of Variance (2 - Way ANOVA)

Source of Variation	SS	df	MS	F	P-value
Between 1 M and 15 M	9613.08	5.00	1922.62	8.84	0.00 *
Time (24, 48 Hours)	8301.27	1.00	8301.27	38.17	0.00 *
Interaction	446.42	5.00	89.28	0.41	0.84
Within	60020.10	276.00	217.46		
Between 10 / 15 x 10^{-3} and 10^{-9} M	18463.65	7.00	2637.67	33.00	0.00 *
Time (24, 48 Hours)	3490.77	1.00	3490.77	43.68	0.00 *
Interaction	614.24	7.00	87.75	1.10	0.37
Within	393.90	80.00	79.92		

Table 32. Effect of Serotonin on *P.viridis* larval settlement

Two way Analysis of Variance (2 - Way ANOVA)

Source of Variation	SS	df	MS	F	P-value
Between 1 M and 3 M	7745.74	1.00	7745.74	31.23	0.00 *
Time (24, 48 Hours)	11079.58	1.00	11079.58	44.67	0.00 *
Interaction	1578.26	1.00	1578.26	6.36	0.01 *
Within	1023.27	28.00	36.55		
Between 1 x 10^{-3} and 10^{-9} M	9345.46	7.00	1335.07	41.35	0.00 *
Time (24, 48 Hours)	10818.01	1.00	10818.01	335.07	0.00 *
Interaction	1797.41	7.00	256.77	7.95	0.00 *
Within	1033.16	32.00	32.29		

Table 33. Effect of Ammonia on *P.viridis* larval settlement

Two way Analysis of Variance (2 - Way ANOVA)

Source of Variation	SS	df	MS	F	P-value
2 - 60 mM	19220.56	9.00	2135.62	56.99	0.00 *
Time (24, 48 Hours)	5847.58	1.00	5847.58	156.04	0.00 *
Interaction	4388.33	9.00	487.59	13.01	0.00 *
Within	1498.98	40.00	37.47		

Table 34. Effect of Copper on *P.viridis* larval settlement

Two way Analysis of Variance (2 - Way ANOVA)

Source of Variation	SS	df	MS	F	P-value
2 - 10 mM	708.29	8.00	88.54	7.97	0.00 *
Time (30 Minutes, 1 Hour)	3308.80	1.00	3308.80	297.69	0.00 *
Interaction	708.29	8.00	88.54	7.97	0.00 *
Within	400.13	36.00	11.12		

Table 35. Effect of Pottasium on *P.viridis* larval settlement

Two way Analysis of Variance (2 - Way ANOVA)

Source of Variation	SS	df	MS	F	P-value
2 - 60 mM	2553.48	9.00	283.72	16.33	0.00 *
Time (24, 48 Hours)	119.99	1.00	119.92	6.91	0.01 *
Interaction	203.83	9.00	22.65	1.30	0.27
Within	694.82	40.00	17.37		

Table 36. Remote settng of *P.viridis* larvae

Two way Analysis of Variance (2 - Way ANOVA)

Source of Variation	SS	df	MS	F	P-value
Percentage Survival (Initial, Final)	58.57	1.00	58.57	9.52	0.00 *
Treatments	341.00	2.00	170.50	27.70	0.00 *
Interaction	3.86	2.00	1.93	0.31	0.74
Within	73.86	12.00	6.15		
Percentage Settlement	22.22	1.00	22.22	1.96	0.19
Treatments	65.33	2.00	32.67	2.88	0.10
Interaction	0.44	2.00	0.22	0.02	0.98
Within	136.00	12.00	11.33		

* significant at $p < 0.05$

4. DISCUSSION

There are two diverging views on mussel larval settlement. Bayne (1965) hypothesized that there is a primary and a secondary settlement phase for the mussel larvae, while Petersen (1984) stated that the larvae settle directly on to mussel beds when competent enough for settlement.

According to the first view, initial settlement of the mussel larvae takes place at a shell length of ~ 260 µm and is generally on filamentous substrates like algae (sea weeds), which allows a period of development free from competition with adult conspecifics (Bayne, 1965; Widdows, 1991) . There follows a variable period of migration which may last upto 3 months during which phase they may attach or detach more than once (Lane *et al.*, 1985). After this migratory phase, which is supposed to reduce competition and predation, permanent settlement occurs on to adult beds or a suitable, previously uncolonized site (Bayne, 1964 *b*, 1976; Seed, 1976; Dare, 1976; Hunt and Scheibling, 1996; Snodden and Roberts, 1997; Vooys, 1999).

Secondary settlers are between 500 µm and 2.5 mm long and no individuals smaller than 500 µm occur on intertidal mussel beds (Bayne, 1965; Dare, 1976; Lane *et al.*, 1985; King *et al.*, 1990). There is also evidence for primary settlement of mussel spat on adult beds (Mc Grath *et al.*, 1988; King *et al.*, 1990).

It is often observed that a film formed by micro – organisms on surfaces has often been called primary biofilms is necessary for the settlement of invertebrate pelagic larvae on any substrate (Zobell and Allen, 1935; Meadows and Campbell, 1972; Crisp, 1974; Weiner *et al.*, 1989; Fitt *et al.*, 1990; Pawlik, 1992; Rodriguez *et al.*, 1993; Unabia and Hadfield, 1999). Conditioning of the substrates or collectors for biofilm formation prior to the settling of mussel larvae has also been tried by some workers both in the field (Kiseleva, 1966; Seed, 1969; Bohle, 1971; Davies, 1974; Kautsky, 1982) and in the hatchery (Bayne, 1965; Petersen, 1984; Van Diepen and Davids, 1986; Satuito *et al.*,1994).

Experiments on primary settlement conducted in this study clearly indicated that except for adults, *P. viridis* larvae settle more on filamentous substrates, mainly byssus threads. This is in partial agreement with other studies in mussel larval settlement where they have found to settle on filamentous substrates other than byssus threads (Blok and Geelan, 1958; Bayne, 1964 a, *b*, 1965, 1976; Hrs-Brenko, 1973 *a*; Tan 1975 *a, b*; Rao *et al.*, 1976; Tortell, 1976; AQUACOP, 1979, 1983; Rengarajan, 1983 *a*; Ceccherelli and Rossi, 1984; Appukuttan *et al.*, 1984, 1988, 1989; King *et al.*, 1989; Widdows, 1991; Lutz and Kennish, 1992; Caceres - Maritnez *et al.*, 1993, 1994; Fuentes and Molares, 1994; Buchanan and Babcock, 1997; Vooys, 1999; Jeffs *et al.*, 1999).

The distinct preference for byssus threads by *P. viridis* larvae over the other filamentous substrates and sea weeds is in contrast to the observations of most of the above reports on mussel larval

settlement. The positive role of byssus filaments on the settlement of mussel larvae in the wild has been reported by few workers (Suchanek, 1981; Davis and Moreno, 1995). Bayne (1976) reported the possibility of byssus thread encouraging the attachment of *M. edulis* larvae in controlled hatchery conditions similar to that observed in nature. Byssal filaments have been found to show a stimulating effect on the settlement of larvae in *P. viridis* by Rengarajan (1983 *a*); in *M eduils* by Blok and Tan – Maas (1977), Eyster and Pechenik (1987); and in *M. galloprovincialis* by Caceres-Martinez *et al.* (1994). Trevelyan and Chang (1983) observed no such attraction in *M. californianus* , while Moreno (1995) and Davis and Moreno (1995) found no positive response in juvenile *Chyromytilus chorsus* for byssal thread extracts from conspecifics.

The exact mechanism of inducement of byssal threads is not known. Waite (1983) believed that induction by byssal threads that are tanned structures, might be related to induction by L – DOPA which is a known component of *M. edulis* byssal threads. Estupinan and Waite (1988) found a two to three fold increase in the settlement and metamorphosis of *M. edulis* larvae by L-DOPA containing polyphenolic proteins extracted from the foot of the adult of this species. Eyster and Pechenik (1987) discounted the theory that inductive chemicals leach out from the byssal threads, which attract larvae to the threads. They, quoting Waite (1983) stated that byssal threads are insoluble in water and hence the question of leaching from the threads into the surrounding sea water medium does not arise at all.

Thus the attraction of *P. viridis* larvae to byssal threads as observed in this study may be due to the chemotactic attraction via a lock and key mechanism as larval foot also contains L – DOPA which is also present in the byssus threads. Purified L – DOPA a tyrosine derivative precursor of dopamine was found to induce settlement and metamorphosis (as observed in this study also and discussed under the effect of L – DOPA on mussel larval settlement) in many commercially important bivalves including mussels (Pawlik, 1990, 1992; Rodriguez *et al.*, 1993).

Appukuttan *et al.* (1984, 1988) found that the larvae of *P. indica* preferred a hard substratum like granite stones to filamentous substrates (like byssus threads). This may be due to the fact that these larvae were of the size range 600 - 700 µm which are definitely secondary settlers, and as literature clearly showed that in related mussel species like *M. edulis* and *P. canaliculus* larvae > 500 µm do not prefer filamentous substrates (Lutz and Kennish, 1992; Caceres-Martinez *et al.*, 1993, 1994; Jeffs *et al.*, 1999).

Bayne (1964 a, *b*, 1976) and Widdows (1991) observed that initial mussel settlement takes place at a shell length of ~ 260 µm on filamentous substrates like sea weeds, which allows a period of development free from competition to 500 – 2500 µm and thereafter they undergo a period of migration to reduce competition and predation (Lane *et al.*, 1985). They finally settle permanently on hard substratum like granite stones, adult beds etc. (Bayne, 1976; Dare, 1976; Seed and Suchanek, 1992; Hunt and Scheibling, 1996; Snodden and Roberts, 1997; Buchanan and Babcock, 1997; Vooys,

1999; Jeffs *et al.*, 1999). Rengarajan (1983 *a*) had clearly indicated the settlement of larvae of *P. viridis* on silk filaments, byssal threads and tiles while settlement in the wild was on lime coated tiles (Rengarajan, 1983 *b*).

Present study indicated that, larvae of *P. viridis* preferred green seaweed *Chaetomorpha antennina* for its primary settlement. Though the percentage settlement on green seaweeds was very poor (4 %) it was far better than the settlement on brown or other red seaweeds. The results of this study are in partial agreement with Hrs-Brenko (1974 *b*) and Ceccherelli and Rossi (1984) who found settlement in *M. galloprovincialis* best on filamentous green algae *Enteromorpha* spp. and poor settlement on oyster shells. Rao *et al.* (1975) had also observed settlement of *M. viridis* seed on green algae *Ulva fasciata*. The present results are in contradiction to the general observations on mussel settlement where larval settlement both in the wild and in the hatchery are good in red algae mostly of the crustose variety (Bayne, 1964 a, *b*, 1965, 1976; Eyster and Pechenik, 1987; Caceres-Martinez *et al.*, 1994). Hickman (1976) found heavy settlement of *P. canaliculus* on floating and drifting brown sea weeds (kelps), while Newell *et al.* (1991) observed good settlement on blades of eel grass (*Zostera marina*).

According to Ramirez and Caceres-Martinez *et al.* (1999), comparisons among efficiencies of filamentous substrates especially artificial ones can give different settlement pattern and results because of their enormous surface variability and their colonization immediately when introduced into seawater which usually does not

happen in controlled hatchery conditions. Further, they were of the opinion that any useful comparison of filamentous substrates would be related to the durability of collectors, handling cost, nature of the study and commercial viability and practical utility. Thus, all the above factors must be looked into when the efficiency of any filamentous substrate is being recommended from a commercial point of view.

Experiments on the effect of adults on the settlement of the larvae of *P. viridis* indicated that larvae settle preferentially on the adults even when the choicest filamentous substrates were available. Similar were the findings of Falmagne (1983) and Petersen (1984) for *M. californianus* and *M. edulis* respectively. They reported that larvae are attracted to adults in controlled conditions. On the other hand, Caceres-Martinez *et al.* (1994) found larvae of *M. galloprovincialis* settling more on byssal threads than on or among the adults kept in the same container. The exact reason for the attraction of the larvae of *P. viridis* towards the conspecific adults is not known although chemical cues emanating from the adults or the larvae themselves have been implicated in other bivalve larval settlement (Pawlik, 1992; Rodriguez *et al.*, 1993).

The results of the effect of temperature on the settlement of *P. viridis* larvae as observed in this study are in partial agreement with other mussel larval rearing experiments of Rao *et al.* (1976), AQUACOP (1979, 1983) for *M. viridis*; Appukuttan *et al.* (1984, 1988) and Sreenivasan *et al.* (1988 *a, b*) for *P. indica* and *P. viridis* respectively. The larval development cycle was faster than what

observed by Tan (1997) who obtained settlement in *P. viridis* by 18-30 days at 27-29 ° C at varying salinities with optimum settlement at 24 ppt by the 18 th day. Results of this study disagree with the observations of Tan (1975 *a*) where quick settlement was observed by 8-12 days at a temperature of 23-25 ° C at 28-30 ppt in *M. viridis* whereas, it took 34 - 41 days in the present study to obtain complete settlement at 24° C in *P. viridis*. Moreover, at 27 ° C in this study, settlement was slower than that reported by Siddall (1979 *a*, *b*, 1980, 1982) (*P. viridis* in 10-12 days at 26.4 ° C and 11-13 days in *P. perna* at 27.7 ° C).

Some authors were of the opinion that higher temperature stimulates setting of *M. edulis* larvae, (Bayne, 1965; Lutz *et al.*1970; Beaumont and Budd, 1982), while others found that low temperature stimulated better settlement (Taylor and Beattie, 1985; Pechenik *et al.*, 1990). There are also reports that temperature has no effect in the setting of *M. californianus* larvae (Trevelyan and Chang, 1983).

The longer duration for completing the settlement at 24 ° C (41 days) in this study is in agreement with the views of Bayne (1983) and Strathman (1987) who were of the opinion that low temperature delay metamorphosis of larvae for longer than at high temperature. Generally it is found that the percentage settlement has a positive correlation with increase in temperature upto a point in mussels *M. edulis* (Bayne, 1965; Hrs-Brenko, 1973 *b*, 1974 *a*, 1978; Sprung, 1984 *a*; Beamount and Budd, 1982; Eyster and Pechenik, 1987; Pechenik *et al.*, 1990) and *M. californianus* in temperate conditions (Trevelyan and Chang, 1983).

In this study moderate aeration enhanced the settlement of *Perna viridis* larvae when compared to heavy or non aerated conditions but only in the presence of a settlement substrate. This is in contrast to the report of Siddall (1982) in the same species where enhanced settlement was recorded in vigorously aerated conditions. Several workers have reported that vigorous aeration is necessary for the increased settlement of other mussel larvae like *M. edulis, M. californianus, M. galloprovincialis* and *P. perna* (Siddall, 1982; Taylor and Beattie, 1985, Eyster and Pechenik, 1987; Velez and Azuage, 1993; Caceres-Martinez *et al.,* 1994), but Trevelyan and Chang (1983) found aeration or increased aeration to be ineffective in the Californian mussel *M. californianus.*

In nature, Appukuttan *et al.* (1989) found heavy settlement of *P. indica* larvae on granite stones lashed constantly by waves. Recently Rajagopal *et al.* (1998 *a*) reported that mussel larvae of *P. viridis* are capable of settling in areas of high water velocity. According to them, this is due to the ability of mussel larvae to withstand high shear force thereby enhancing the propagule flux ratio to the substratum.

It is believed that the higher settlement in heavily aerated conditions was not due to increased oxygen concentrations in aerated cultures but due to increased frequency of contact between the larvae and the substratum, and moreover water circulation and agitation may help in dispersing localized attracting factors for settlement to a wider area thereby attracting more number of larvae to the substratum (Walne, 1974; Eyster and Pechenik, 1987). Poor

settlement in aerated cultures without settlers as observed in the present study is in agreement with Bayne (1965) who noticed that *M. edulis* larvae tend to delay settlement when the competent pediveliger larvae are not provided with suitable substratum. Mussel larvae have been reported to have prolong larval life for months in adverse conditions (Bayne, 1983; Sprung, 1984 *a*; Pechenik *et al.*, 1990).

Results of the present study showed that *P. viridis* larvae did not have light preference (300 Lux) even though the percentage of settlement was slightly better in dark when compared with lighted conditions after 48 hours. This is in agreement with Bayne (1964 *a*) who observed that *M. edulis* larvae respond differently during the various stages of light intensity (0 – 3300 Lux), they were photo negative and settle better in dark or shaded conditions. He also indicated that light intensity is affected by temperature. On the other hand in *P. perna* larvae poor settlement in > 30 Lux light intensity was observed by Velez and Azuaje (1993).

P. viridis larvae prefer to settle more on black rather than white substratum followed by red, blue, green and yellow, in the order of maximum settlement. In experiments where only the bottom part of the tank was given the respective colour, the results were better than fully covered ones indicating that ambient light also has a role in colour choice. Similar results were observed by Tortell (1976), who found that black fibrillated polypropylene film attracted more *P. canaliculus* larvae than films of other colours.

Though it is assumed that invertebrate larvae have no true colour vision, discrimination of colours, if any, would be based on the reflected light intensities, where colours at red reflective appear dark and those near blue - green appear light (Yule and Walker, 1984). Hence, the preference of *P. viridis* larvae to settle on dark coloured substrates like black and red and poorly on blue and green, may be due to this reason.

P. viridis larvae are able to settle in a wide range of salinity regimes (15 - 45 ppt) even though these extremes are not conducive for their growth and survival (< 20 ppt). Best settlement was obtained in salinity regimes of > 25 ppt and poor in salinities above 40 and below 25 ppt. Literature on the effect of salinity on mussel larval settlement is meager even though Bayne (1965) had mentioned about delay in *M. edulis* settlement and metamorphosis at suboptimal salinity levels. The results of the earlier larval rearing and that of settlement at different salinities showing that mussel larvae settle at mid to high saline ranges is a confirmation of the observations on *P. viridis*. Rajagopal *et al.* (1997, 1998 *a, b*) found settlement of *P. viridis* larvae correlated with high temperature and high salinity with peak in May to June when the values of both these parameters are very high in the wild. Nair and Nair (1985) have also emphasized that moderate to high salinity (24 - 33 ppt) was the main factor and not temperature in the settlement of the oyster *Crassostrea madrasensis* in Cochin backwaters.

P. viridis larvae settled in a wide range of pH from 6 - 9 and the settlement was best in neutral (7) to alkaline pH 8.2 after 48

hours and nearly 50 % in pH 6 and pH 9. After 96 hours, high mortality occurred in pH 5 and 9 while larvae were healthy in 6, 7 and 8. This is in concurrence with the findings of Bayne (1965) for the larvae of *M. edulis* which were found to settle in 7 – 8 pH. Clam *M. mercenaria* and oyster *C. virginica* larvae have also been reported to settle in a wide range of pH 6.75 - 8.75 but growth and survival was less above and below this range as no settlement occurred (Loosanoff and Davis, 1963). pH of 7 – 9 had no inductive effect for *C. virginica* larval settlement (Prytherch, 1934).

L-DOPA 2.5 x 10^{-7} M from a wide range of concentrations 1- 15 x 10^{-3} - 10^{-9} M has been found to induce 100% settlement within 5 hours in *P. viridis* larvae. L-DOPA has been able to induce *M. edulis* larvae to settle and metamorphose at 1 x 10^{-5} M in 24 - 40 hours (Cooper 1982, 1983) and in *M. galloprovincialis* (Satuito *et al.*, 1999) at 10 x 10^{-6} M in 24 hours.

Since the larvae exposed to L-DOPA exhibit natural settling behavior, the sources of L-DOPA or L-DOPA mimetic molecules which result in complete settlement and metamorphosis, could be bacterial films, juvenile of adult oysters or their pheromones, byssus threads, mussel foot or a combination of all these factors (Crisp, 1967; Veitch and Hidu, 1971; Waite, 1983; Coon *et al.*, 1985, 1990 *b*; Estupinan and Waite, 1988; Pawlik, 1990; Weiner *et al.*, 1989; Walch *et al.*, 1999). It is still not clear how L-DOPA induces settlement in bivalve larvae, though various views, pathways and

models have been put forward (Burke, 1983; Bonar *et al.*, 1990; Coon *et al.*, 1990 *a*).

Much of the work with γ - Amino Butyric Acid (GABA) has been carried out on gastropod species (*Haliotis* spp.) where it was shown to have a highly stimulative effect, and settlement occurred between $1 \times 10^{-5} - 10^{-6}$ M concentration of GABA, with best settlement at 1×10^{-6} M (Morse *et al.*, 1979 *a, b*; Morse, 1990, 1992 ; Bryan and Qian, 1998; Searcy – Bernal and Anguiano - Beltran, 1998). However, in bivalve molluscs GABA ($1 \times 10^{-4} - 10^{-6}$ M) has been reported to have little or no effects in *M. edulis* (Cooper, 1982; Eyster and Pechenik, 1987), *Perna viridis* (Baylon, 1988), *Crassostrea gigas* (Coon *et al.*, 1988; Beiras and Widdows , 1995 *a*) and *C. hastata* (Hodgson and Bourne, 1988). In all these studies, the larvae were swimming even after 24 hours, but Tan and Wong (1995) found significant improvement in the percentage settlement and metamorphosis in the tropical oyster *C. belcheri* when effected by $10^{-4} - 10^{-6}$ M GABA.

According to Morse *et al.* (1979 *a, b*), prolonged exposure to GABA can cause habituation of the larvae. Hence, whether the induction of *P. viridis* larvae after 24 or 48 hours is due to their normal physiology or to the inductive effect of the chemical is not clear.

P. viridis larvae failed to settle in all Serotonin concentrations from 1×10^{-4} to 10^{-9} M even after 24 hours. Moreover, 3×10^{-3} to 10^{-9} M concentration was found to be inhibitory for settlement of

P. viridis larvae as percentage settlement in this concentration was only half that of 1 x 10^{-3} to 10^{-9} M concentrations.

In mussels, Satuito *et al.* (1999) found 100 µ M of Serotonin was effective only to some extent in inducing settlement and metamorphosis in *M. galloprovincialis* when other neurocompounds were better effective at 1/10 th of Serotonin's concentration. Moreover, continuous exposure for 7 hours at 3 x 10^{-5} – 10^{-9} M Serotonin causes abnormal larval behaviour in *M. edulis* larvae (Beiras and Widdows, 1995 *b*). The results of Serotonin as an inducer of settlement and metamorphosis of invertebrate larvae are varying, and in the case of bivalve molluscs it is a poor inducer of settlement and metamorphosis.

The percentage settlement of *P. viridis* larvae increased when exposed to different millimolar concentrations of ammonia with increasing concentration upto 10 mM and then decreased at higher concentrations of 30 and 60 mM. Larvae settled best in 10 mM (82%) after 48 hours. On the contrary, Coon *et al.* (1990 *b*), Bonar *et al.* (1990) and Kingzett *et al.* (1990) observed immediate settlement of larvae within 30 minutes in *C. gigas*, *C. virginica* and *Pecten yessoensis* respectively at 2.5 mM. The present study revealed that the concentration of ammonia required for *P. viridis* settlement was four times (10 mM) more than the concentration mentioned by the above workers. The failure of the larvae to settle within 30 minutes or 5 hours as observed in this study shows that either the larvae had been habituated to the test concentrations or the larvae have not been affected by these test concentrations.

The exact mechanisms by which larvae settle due to ammonia are not known even though several authors have postulated that it increases intracellular alkalization (increase of intracellular pH) or regulates the intracellular transmethylation (Berking, 1988; Coon *et al.*, 1988, 1990 *b*).

When exposed to millimolar concentrations of copper, the mussel larvae were found to settle within half an hour. Prytherch (1931, 1934) and Nell and Holliday (1986) made similar observations for the larvae of *C. virginica* and *Saccostrea commercialis* respectively.

In his exhaustive work with *C. virginica* larvae, Prytherch (1931, 1934) found that 0.5 ppm or less, copper concentrations are non toxic even when they were exposed continuously. He further stated that concentrations of 0.8 - 2.5 ppm also were toxic if the larvae remained in this solution continuously but if removed (2-3 minutes) after the first exposure then normal spat was formed and concentrations of copper above 3 ppm for 3-5 minutes were fatal for the larvae (Peytherch 1931, 1934).

Thus the complete mortality which took place within 5 hours in this study could be due to the concentration used in the study (1-10 ppm) and the duration of the exposure. However, in a similar study with the oyster *S. commercialis*, Nell and Holliday (1986), observed mortality of the larvae only after 5 days of exposure.

P. viridis larvae was found to settle in a threshold concentration of 30 mM (from a tested range 2 – 60 mM) but only

50 – 55 % settled from 12 – 48 hours and the settlement was poor in high concentrations. In contrast to this study, Eyster and Pechenik (1987) indicated that even with appropriate filamentous substrates and an elevated K^+ concentration from 5 – 20 mM failed to induce settlement in *M. edulis*. Elevated K^+ concentration was found not be particularly inductive for bivalve larvae especially oysters (Nell and Holliday, 1986; Beiras and Widdows, 1995 *a*). Martinez *et al.* (1999) found metamorphosis percentage increased upto 48 hours in scallop *Argopecten purpuratus* larvae.

The failure of the larvae to settle upto 12 hours in the present study may be due to the fact that excess of potassium is dose dependent and at an optimal concentration the stimulus must be provided continuously for at least 20 hours for complete settlement and metamorphosis to occur. Withdrawal of the stimulus prior to this time results in only temporary attachment and the very high concentrations of the chemical is toxic to the larvae (Baloun and Morse, 1984; Yool *et al.*, 1986; Beiras and Widdows, 1995 *a*). Hence the reduced percentage of settlement and mortality of the larvae at high concentrations > 30 mM in this study could be due to this reason.

Though remote setting has also been successfully demonstrated in mussels *M. edulis* (Trevelyan, 1989) and *M. galloprovincialis* (Kupier, 1991) details on survival and settlement were not mentioned. It was observed that survival and settlement of *P. viridis* larvae transported in moist packages in ambient temperature was only slightly inferior to the control. However, in

contrast to the present study, the observations of Henderson (1982) and Panggabean *et al.* (1989) on *C. gigias*; Holliday *et al.* (1991) on *C. gigas* and *S. commercialis*; and Unnikrishnan *et al.* (2000) on *C. madrasensis* about remote setting showed that the percentage survival and settlement of remotely set larvae were better when they were held or transported at low to very low temperatures.

Irrespective of the treatment, the pre and post set percentage settlement obtained in this study are comparable to similar work on other bivalve larvae by Unnikrishnan *et al.* (2000) in *C. madrasensis* and superior to the results obtained by Burnell *et al.* (1993) and Roland *et al.* (1989) in *C. gigias* and Tan and Wong (1996) in *C. belcheri* where they reported 17 – 30 % settlement.

Thus, it could be said that for the remote setting process to be initiated in *P. viridis*, the presence of a well-developed foot could be a more appropriate indication on the competence of the mussel larvae for settlement. This is contrary to the proposal of shell length and eye spot diameter as criteria to decide the competency for the remote setting of the larvae by Holliday *et al.* (1991).

Roland *et al.* (1989) observed that the proportion of the larvae setting was affected by factors like salinity, temperature, aeration, feeding and type of cultch material. Thus the percentage settlement in this study could have been increased by optimizing these parameters or by the use of settlement inducing chemicals like L – DOPA. Hence, remote setting technique could probably be

attempted with further experiments and can be used as a potential tool for the popularization of bivalve farming in India.

SUMMARY

SUMMARY

From the point of view of development of a commercial hatchery technology for green mussel *Perna viridis*, studies were undertaken on the induced maturation, spawning and larval rearing and settlement of this species. The summary of the results is given below.

Perna viridis broodstock could be conditioned or induced to mature out of the spawning season by maintaining them at 23 ± 1 ° C, at > 30 ppt salinity, pH 7.5 – 8.2 and fed with *Chaetoceros calcitrans* at a cell concentration of $1x\ 10^{6}\ ml^{-1}$ fortified with 700 I.U. cod liver oil @ $1\ l\ animal^{-1}\ day^{-1}$ in two installments for 15 and 32 days respectively. The percentage of spawning and day taken to spawn decreased when fortified (non fortified *C. calcitrans* fed animals at 23 ± 1 ° C spawned on the 50th day) but the results among these two diets were not significant.

Studies on salinity and pH showed that *P. viridis* fail to mature in salinity less than 20 ppt. The time to mature and spawn increased with decreasing salinity and increasing water temperature. pH in the range of 7 – 8 did not significantly affect the maturation process.

Induced spawning experiments showed that the threshold limits of salinity and pH was 25 – 40 ppt and 7 – 8.2 respectively with better spawning in higher ranges in both these parameters.

Temperature stimulation significantly increased the percentage spawning but only within the earlier mentioned threshold limits of salinity and pH. Addition of male / female gametes along with a thermal stimulation (+ 5 ° C from the ambient temperature) gave nearly 100 % spawning in *P. viridis* and this was the best treatment for the induced spawning of *P. viridis*.

None of the chemicals like H_2O_2 and Tris were able to elicit a full spawning response in *Perna viridis*. The mussels spawned partially or did not spawn in any of the chemical treatments.

Studies on the effect of physico – chemical parameters for larval rearing of *P. viridis* showed a temperature of 29 ° C – 31 ° C at high salinity > 30 ppt with 50 ppm Chloramphenicol antibiotic at pH 8.2, with moderate aeration and fed with *Isochrysis galbana,* as the best for optimum growth, percentage survival and settlement of spat.

Settlement preference indicated clearly that primary settling larvae required filamentous substrates and among these byssus threads was the best substrate.

Among the seaweeds used for settlement experiments, green seaweed *Chaetomorpha antenina* was the best for the settlement of *P. viridis*.

Presence of adult mussels in the rearing medium showed enhanced settlement of *P. viridis* larvae better than the settlement obtained on byssal threads.

Temperature induced the settlement of *P. viridis* larvae. High temperature of 31° C was better for settlement than lower temperatures (29 ° C, 27 ° C and 24 ° C)

Moderate aeration or no aeration has shown to be the best for *P. viridis* larval settlement but settlement was delayed if no substrates were available even in aerated conditions.

Light did not have any influence on the settlement of *P. viridis* larvae.

Data indicated that different colours had no significant influence on the settlement of mussel larvae. Extreme colours white and black were significantly better for the settlement of *P. viridis* larvae when compared to other colours though they did not differ significantly among themselves, Among the other colours, red was the best and yellow the worst.

Effect of salinity on mussel larval settlement indicated that mussel larvae are able to settle in a wide range of salinity regimes (15 to 45 ppt), some of which are not favorable for its early larval growth and survival ie., extreme salinities of 15 ppt and 45 ppt at ambient water temperature of 31 ° C. Salinity of 25 – 38 ppt was the best for the settlement of *P. viridis*.

The effect of pH showed that a pH range of 7 - 8 was the best for the settlement of *P. viridis* larvae.

L - DOPA gave the best results among the chemicals with 100 % settlement observed in 5 hours at 2.5×10^{-7} M.

GABA was effective only in very high dosage 10 – 15 $\times 10^{-6}$ M after 24 hours but there was subsequent mortality. In all other doses settlement was best in the range 10^{-5} - 10^{-6} M. For Serotonin, 1 M was better than 3 M. Settlement was high in the highest concentrations but did not show any particular pattern. 1 M was inductive in high concentrations of 1×10^{-3} - 10^{-4} M in 5 hours but in 1×10^{-5} - 10^{-9} M settlement was observed only after 24 hours. Here too like GABA, total mortality of the larvae was observed in high concentrations.

Ammonia at 10 mM at 48 hours was the best for the settlement of *P. viridis* larvae.

Potassium at 30 mM at 48 hours was the best for the settlement of *P. viridis* larvae

Among all the chemicals used, immediate settlement was observed in copper which was effective in inducing settlement of 80 % *Perna viridis* larvae in less than half an hour in all the concentrations (2 - 10 mM) tested, however, irrespective of the concentration all the larvae died.

Remote setting of *P. viridis* was successfully employed. The percentage survival and settlement of larvae transported at ambient temperature (31 ° C) in moist packaging was only slightly inferior to the larvae transported in water and larvae transported in moist

packages at low temperature (25 ° C) performed poorly. A 24 hour transportation period did not significantly affect the percentage survival or settlement of *P. viridis* larvae and hence can be used.

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