2. MATERIALS AND METHODS

2.1. Experimental animal

Systematic Classification - *Artemia species*

Phylum	:	Arthropoda
Class	:	Crustacea
Order	:	Anostraca
Family	:	Artemidae
Genus	:	*Artemia*
Species	:	*franciscana*

2.1.1. Biology

2.1.1.1. Habit and Habitat

Artemia is an euryhaline animal found abundantly in athalassohaline and thalassohaline environments at salinity levels ranging from 10g/L(Agh *et al.*, 2007) to 340g/L (Post and Youssef, 1977).,

2.1.1.2. Distribution

Populations of *Artemia* are found in more than 600 habitats distributed across the world in salt lakes, natural and man-made salterns (Van Stappen, 2002). The distribution of *Artemia* is discontinuous, not all highly saline biotopes are populated with *Artemia*. Their distribution reflects the flight paths of migratory birds (Brochet, *et al.,* 2010 b; Sanchez *et al.,* 2012; Munnoz *et al.,* 2013; Viana *et al.,* 2013, Varo *et al.,* 2011, Vest and Canova 2011) and deliberate inoculations for commercial purposes by man (Persoone and Sorgeloos, 1980). In India, natural populations of

Artemia have been found in the salt farms of Maharastra, Rajasthan, Tamilnadu and Gujarat. Nearly 19 potential *Artemia* sites have been identified in India with ten in Tamilnadu.

2.1.1.3. Adaptation

The brine shrimp thrive well in natural sea water. They cannot migrate from one saline biotope to another via the seas, as they depend on their physiological adaptations to avoid predation and competition with other filter feeders. Its physiological adaptations to high salinity are very efficient osmoregulatory system, the capacity to synthesize very efficient respiratory pigments to cope with the low oxygen levels at high salinities and the ability to produce dormant cysts when environmental conditions endanger the survival to the species. Therefore*Artemia* is only found at salinities where its predators cannot survive (Van Stappen, 2002).

2.1.1.4. Feeding

Artemia is non-selective filter feeders of organic detritus, microalgae as well as bacteria (Van Stappen, 2002). The continuous beating of the thoracopods exhibited by the animal for respiration helps to collect food particles. *Artemia* seems to regulate the food intake as the function of chemical composition of the diet. The food intake also depends on the retention time in the gut, enzymatic activity and digestability of the food (Sorgeloos *et al.*, 1986).

2.1.1.5. Growth

Artemia starts feeding from II instar after 12h hatching. It reaches adult in two weeks, increasing in length by a factor 20 and biomass by a factor 500 (Sorgeloos *et al.*, 1986). Matured within two weeks and has the life span of about six months.

2.1.1.6. Reproduction

Artemia species is zygogenetic both males and females are present in the population. The male has a pair of very distinctive muscular graspers (2^{nd} antennae) in the head region, whereas in the posterior part of the trunk region a pair of penis can be observed. Female *Artemia* have no distinct appendages and are recognized by the brood pouch (or) uterus, which is situated just behind the 11^{th} pair of thoracopods. Eggs develop in two tubular ovaries situated in the abdomen. Once ripe, they become spherical and migrate via two oviducts into the unpaired uterus. Precopulation in adult *Artemia* is initiated by the male when grasping the female with his hooked antennae in between the uterus and the last pair of thoracopods. Couples can swim around for long periods in their so called 'riding position', beating their thoracopods at a synchronous rate. Copulation itself is a reflex: i.e. the male abdomen is bent forward, one penis is introduced into the uterus aperture and the eggs are fertilized.

After fertilization, the eggs normally develop into free swimming nauplii (Ovoviviparous reproduction), which are set free by the female. In extreme conditions, the eggs are surrounded with a touch brown shell. The egg is then called as a cyst. The cysts are released by the female in the water. This is called oviparous reproduction. The colour of adult *Artemia* is correlated with the mode of reproduction, because haemoglobin synthesis is activated by low oxygen concentration in the water. Red *Artemia* indicates oviparous reproduction while pale whitish *Artemia* indicate ovoviviparous reproduction.

The cyst released by the female is in a state of metabolic dormancy. The cysts usually float in the high salinity waters and are blown ashore where they accumulate

and dry. As a result of this dehydration process, the diapause mechanism is inactivated allowing the cyst to resume their further embryonic development when hydrated in optimal hatching conditions.

2.1.1.7. Life history

The life cycle begins from instar I nauplius stage onwards. The nauplii are delivered during favourable conditions through ovoviviparous mode of reproduction or the nauplii come from the cysts produced during unfavourable conditions through oviparous mode of reproduction.

2.1.1.7.1. Nauplius

The first nauplius stage (instar-I) measures 400 – 500 microns in length, brownish orange colour and has three pairs of appendages i.e. the antennae, the antennules and the mandibles.

2.1.1.7.2. Metanauplius

The metanauplius has four stages. Food ingestion started in the metanauplius stage I small food particles (algal cells, bacteria and detritus) ranging in size from 1 to 40 µm are now being ingested into the functional digestive tract. At the end of metanauplius stage IV, the compound eyes are developed. The first pair of thoracopds developed exopodites and endopodites, protopodites appeared in the second and third pairs of thoracopods and the fifth and sixth pairs of thoracopods appeared.

2.1.1.7.3. Post larvae

There are seven post metanauplius stage. During this stage, the thoracopods attained their full development. The abdominal segment VIII and telson appeared.

The buds of the ovisac appeared in the ventral side of the two genital segments. During post larvae (Juvenile) stage, the eye stalk attained the maximum extension and the ovisac attained the full expansion.

2.1.1.7.4. Adult

Artemia requires about 14 moults to reach its terminal size and achieves sexual maturity in about 12 moults. The adult *Artemia* has five pairs of appendages *viz*. antennae, antennules, mandibles, and maxillae were present in the cephalic region. The thorax consisted of 11 segments (thoracomeres) provided with one pair of thoracopods each. The thoracopods were multifunctional phyllopods showing division of labour such as locomotion, food gathering, osmoregulation and respiration. The abdomen was provided with eight segments and the telson. In each furcal ramus of the telson 12 or 15 feathered setae were present.

2.2. Test organism

Brine shrimp *Artemia* cysts were purchased from Chemical laboratory, Nagercoil and were certified to be *Artemia franciscana*, were used as a test organism. The cyst was stored in dark at 5^{o}C until used for testing.

In the present study,the acute and chronic toxicity of *Artemia sp.* in response to pesticides, organochlorine (Lindane, Endosulfan), organophosphate (Monocrotofos, Malathion), pyrethroid (Fenvalerate, Cypermethrin), carbamate (Carbaryl, carbofuran) were studied. Before studying bioassay, characterization of *Artemia* was studied.

2.2.1. Biometrics of cysts

The cysts were hydrated in fresh water for two hours and the diameter of a random samples of hundred spherical cysts with five replicates were measured using a compound microscope equipped with an ocular micrometer, and the average was calculated.

2.2.1.1. Decapsulation of the cyst

The cysts were hydrated for one hour and treated with decapsulation solution containing 0.7g bleaching powder and 0.67g sodium carbonate in 14 ml of seawater. The cysts were kept in suspension by bubbling air from the bottom. A gradual colour change from dark brown to grey indicated the dissolution of the chorion. After 15 minutes, when there was no colour change, the cysts were washed thoroughly in water until chlorine smell was lost. The cysts were dipped in 1N HCl for the deactivation of active chlorine residues and washed in water.

2.2.1.2. Measurement of diameter of cysts

The diameter of the hydrated and decapsulated cysts were measured using a compound microscope equipped with an ocular micrometer Vanhaecke and sorgeloos (1980).

2.2.1.3. Determination of chorion thickness

The chorion thickness was calculated after decapsulation (Sorgeloos *et al.*, 1986) by using the following formula

$$\text{Chorion thickness} = \frac{\text{Diameter of the hydrated cysts} - \text{Diameter of the decapsulated cysts}}{2}$$

2.2.1.4. Hatching of cyst

Commercially available brine shrimp *Artemiafranciscana* cysts were used in toxicity studies. The cysts were hydrated in freshwater for two hours and were transferred to a narrow glass vessel containing natural seawater. Continuous moderate aeration was given from the bottom to keep the cysts moving. The cysts were hatched under constant illumination (1000 lux) at 28 ± 1^0C.

2.2.1.5. Removal of larvae from the hatching medium

The nauplii were separated from the empty shells and unhatched cysts by taking advantage of the positive phototactic behaviour of the larvae. The contents of the hatching vessel were poured into a glass bowl. It was covered with a black paper with a hole in the centre. A light beam was directed through the hole. Attracted by light, the larvae crowding under the hole were sucked out with the help of a Pasteur's pipette and transferred into the culture medium.

2.2.1.6. Length of nauplii

The hatched nauplii were removed from the hatching medium and fixed in iodine tincture. A random sample of 100 nauplii (with 5 replicates) length was measured with a compound microscope equipped with an ocular micrometer.

2.3. Rearing conditions

2.3.1. Rearing medium

Salinity of 35ppt was prepared by dissolving salt, which is used as a rearing medium. A hand salinity refractometer (NEWS-10, TANAKA SANJIRO-Japan) of 1 ppt sensitivity was used to check salinity. 10 larvae were reared in 100 ml of brine in

250 ml glass bowls. The culture medium was changed once in four days. The salinity of the medium was checked every morning and evening. The volume and salinity of the medium were kept constant.

2.3.2. pH (Hydrogen ion concentration) of the medium

The pH of the rearing medium was maintained at 8 using sodium bicarbonate. The pH of the medium was measured with water proof pH scan – 2 (Made in Singapore).

2.3.3. Temperature of the medium

Temperature of the medium was recorded every morning and evening with the thermometer (Hermes, India, Sensitivity1^{0}C). The experiments were done in ambient temperature (28 ± 2^{0}C).

2.3.4. Dissolved oxygen of the medium

The experimental medium was moderately aerated and the oxygen content was maintained at 3.5 ± 0.5 mg/l. The oxygen content was determined by Winkler's method (APHA, 1985)

2.3.5. Feed

Artemia is non – selective filter feeder (Reeve, 1963) filtering particles less than 50µ in size (Dobbeleir *et al.*, 1980). Utilization of various artificial diets, algae or yeast is always complicated with the contamination of some undesired chemical substances or metabolites. Hence Artemia is treated with 1% solution of glucose, used as a source of energy (Dvorak *et al.*, 2005).

2.3.5.1. Preparation of the feed

Glucose powder was purchased from the market. 1gm of glucose is diluted with 100ml of distilled water to make 1% glucose. It was stored in refrigerator. *Artemia* were fed twice daily (approximately 0.5ml /100ml of the medium). Before feeding the dead individuals and other residues were removed from the medium.

2.4. Morphometric and Meristic characteristics of adults

Ten morphometric characters which are common between males and females measured (Asem and Rastegar-Pouyani, 2007; Zhou *et al.*, 2003; Camargo *et al.*, 2003; Amat *et al.*, 2005). Adult *Artemia* of fully developed males and females were fixed in iodine tincture and the ten morphological characters were studied.

The total length (TL) and abdominal length (AL) were measured using a dissection microscope with a graph paper fixed on the platform. The abdominal width (AW), furcal length (FL), head width (HW), eye separation (ES), diameter of the eye (ED), length of the first antenna (LA) and ovisac width (OW) were measured with a compound microscope, the eye piece of which was equipped with a micrometer (Amat, 1980; Basbug and Demirkalp, 1997). The number of setae on the left branch (LS) and right branch (RS) of the furca was counted with a compound microscope.

2.5. Toxicology

Recent toxicology has demanded decrease in the number of vertebrates used in toxicology testing and their partial replacement with invertebrate animals. During the last 50 years, various invertebrate species have been tested for their sensitivity to many chemical or physical agents. The most valuable organism available for

ecotoxicity testing belongs with crustaceans of the *Artemia* genus commonly known as brine shrimp (Dvorak *et al.,* 2012).

The *Artemia* species have been found convenient for various short or long term toxicity testing. The most important of them is the lower sensitivity of the *Artemia* species to several physical or chemical agents in comparison to the other invertebrate test organisms (Sorgeloos *et al.,* 1978; Song and Brown, 1998; Okamura *et al.,* 2000; Guerra, 2001; Nalecz-Jawecki *et al.,* 2003; George-Ares *et al.,* 2003; Mayorga *et al.,* 2010).*Artemia* offer many advantages to favour them as convenient for the standard testing in ecotoxicology, high adaptability to variety of testing conditions, high fecundity, bisexual versus parthenogenetic strategies, small body size, varied nutrient sources, high hatchability, simple availability and low cost of tests.

2.5.1. Pesticides

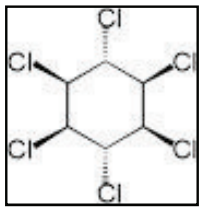
The pesticides used in the present study were Lindane and Endosulfan (Organochlorine), Monocrotofos and Malathion (Organophosphate), fenvalerate and cypermethrin (pyrethroid), carbaryl and carbofuran (carbamate). In the present study, the effect of pesticides on *Artemia franciscana* was studied.

2.5.1.1. Organochlorine

These are considered as synthetic organic pesticides that were discovered and used earlier. Organochlorine pesticides are a large class of multipurpose chlorinated hydrocarbon chemicals. Organochlorine pesticides break down slowly in the environment and accumulate in the fatty tissues of the animals (Swackhamer and Hites, 1988). Prolonged use in large quantities will easily lead to environmental

pollution and accumulation in mammals resulting in cumulative poisoning or damage. Many organochlorine pesticides are endocrine disrupting chemicals, meaning they have subtle toxic effects on the body's hormonal systems (Lemaire *et al.*, 2004). Exposure to low concentrations of organochlorine chemicals over a long period may eventually lead to a substantial body burden of toxic chemicals (Quintana *et al.*, 2004). Chemicals in a maternal body burden may be transferred to foetus to the placenta and to children through breast feeding (Jurewicz and Hanke, 2008). Organochlorine pesticides are carried long distances via atmospheric and oceanic currents from where they are manufactured and used, and build up in the fatty tissues of animals (Bentzen *et al.*, 2008). Many studies have linked organochlorine pesticide exposure milk consumption of contaminated animal products, mostly meat, dairy, fish and marine animals (Fitzgerald *et al.*, 2001; Hagmar *et al.*, 2001; Mwevura *et al.*, 2002; Bradman *et al.*, 2007).They are excellent examples of pesticides with numerous documented negative health impacts.

2.5.1.1. a. Lindane

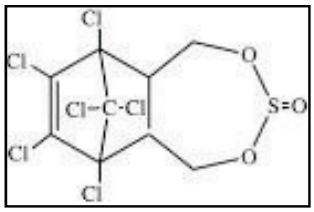


Lindane is also known as gamma – hexa chlorocyclohexane (r-HCH), gammaxene, Gammallin and erroneously known as benzene hexachloride (BHC) (Brandenberger *et al.*, 1997). It is an organochlorine chemical variant of hexachlorocyclohexane that has been used both as an agricultural insecticide and as a pharmaceutical treatment for lice and scabies (Lawson *et al.,* 2011).

Lindane is a neurotoxin that interferes with GABA neurotransmitter function. In humans, lindane affects the nervous system, liver and kidneys and may be a

carcinogen. Lindane is a persistent organic pollutant in the environment. It is transported long distances by natural processes like global distillation, and it can bioaccumulate in food chains, though it is rapidly eliminated when exposure is discontinued (Gianessi and Leonard, 1999).

2.5.1.1. b. Endosulfan



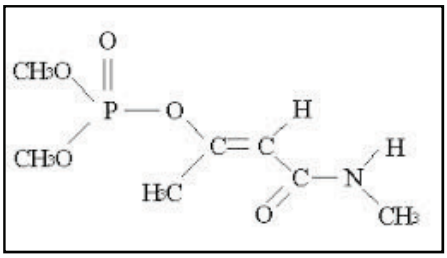
Endosulfan sulfate is a product of oxidation containing one extra O atom attached to the S atom. Endosulfan has been used in agriculture all around the world to control insects' pests. Due to its unique mode of action, it is useful in resistance management; however, it can negatively impact on beneficial insects. Besides, it is one of the most toxic pesticides on the market today, responsible for many fatal pesticide poisoning incidents around the word. Endosulfan can act as an endocrine disruptor, causing reproductive and developmental damage in both animals and humans. Endosulfan is acutely neurotoxic to both insects and mammals including human beings (Chakraborthy *et al.,* 2010).

Besides, it is a ubiquitous environmental contaminant. The chemical is semi-volatile and persistent to degradation processes in the environment. Endosulfan is subject to long range atmospheric transport, it can travel long distances from where it is used. More than 80 countries including the European Union, Australia, several West African nations, the United States, Braziland, Canada had banned it. It is still used extensively in India, China and few other countries (Hong *et al.,* 2008).

2.5.1.2. Organophosphate

Organophosphates (OP) are derivatives of phosphoric acid, OP have replaced the banned organochlorine compounds and are a major cause of animal poisoning. They vary in toxicity, residual levels, and excretion. Many have been developed for plant and animal protection.Organophosphate pesticides degrade rapidly by hydrolysis on exposure to sunlight, air and soil, although small amounts can be detected in food and drinking water. Although organophosphates degrade faster than the organochlorides, they have greater acute toxicity, posing risks to people who may be exposed to large amounts.

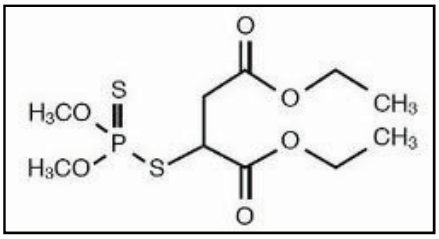
2.5.1.2. a. Monocrotophos



Monocrotophos is an organophosphate insecticides chemical name is Dimethyl-1 methyl-2 vinyl phosphate. Monocrotophos affects the nervous system by inhibiting acetylcholinesterase, an enzyme essential for normal nerve impulse transmission. Monocrotophos can be absorbed following ingesion, inhalation and skin contact. Monocrotophos is not usually detected as a residue in food in total diet (Chowdhury *et al.,* 2012).

Monocrotophos has a low environmental persistence. It does not accumulate in soil because it is biodegradable. Monocrotophos and its metabolites are not expected to bioaccumulate. Monocrotophos is moderately toxic to fish. Monocrotophos is acutely toxic to birds and humans. It may also kill birds which eat insects poisoned with monocrotophos. Monocrotophos was cited in the death of 23 school children ate the state provided school lunch in the district of Saran in India in July 2013.

2.5.1.2. b. Malathion

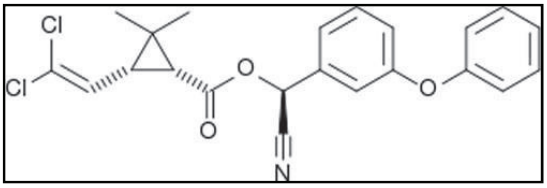


Malathion is an organophosphate para sympathomimetic which binds irreversibly to cholinesterase. Malathion is an insecticide of relatively low human toxicity. Malathion is a pesticide that is widely used in agriculture, residential landscaping, public recreation areas, and in public health pest control programmes such as mosquito eradication. Malathion in low doses is used as a treatment for head lice, body lice and scabies (Chakraborthy *et al.,* 2010).

2.5.1.3. Pyrethroids

Pyrethrins are widely used for control of various insect pests. Pyrethroids are synthetic forms of pyrethrins synthetic pyrethroid insecticides have been used for more than 20 years to control insect pests in a variety of crops (Maund *et al.*, 2001). Pyrethroids offer low toxicity to human applicators and non-target mammals and birds, they are highly toxic to invertebrates and also fishes (Palmquist *et al.*, 2010). Degradation of pyrethroid insecticide was observed to be more rapid in natural versus sterilized soils indicating that biological processes to contribute to breakdown in soil.

2.5.1.3. a. Cypermethrin

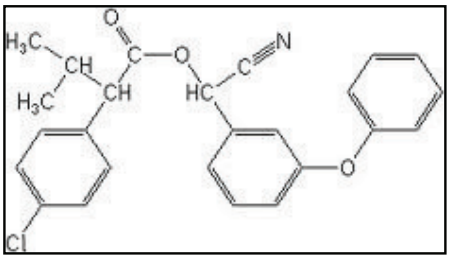


Cypermethrin is a synthetic pyrethroid used as an insecticide in large scale commercial agricultural applications as well as in consumer products for domestic purposes. It behaves as a fast acting neurotoxin in insects. It is easily degraded on soil and plants but can be

effective for weeks when applied to indoor inert surfaces. Exposure to sunlight, water and oxygen will accelerate its decomposition (Gattuso, 2000).

Cypermethrin is highly toxic to fish, bees and aquatic insects. It is found in many household ant and cockroach killers including raid and ant chalk. Excessive exposure in human can cause nausea, head ache, muscle weakness, salivation, shortness of breath and seizures. Cypermethrin kills beneficial insects and animals as well as the targeted insects. Fish are particularly susceptible to cypermethrin (Stephenson, 1982). Resistance to cypermethrin has developed quickly in insects exposed frequently and can render it ineffective (Martinez-Cabrillo *et al.*, 1991).

2.5.1.3. b. Fenvalerate



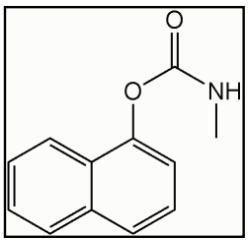
Fenvalerate is a pyrethroid insecticide. It is considered as an insecticide of moderate mammalian toxicity. In laboratory animals, central nervous system toxicity is observed following acute or short term exposure. Fenvalerate has applications against a wide range of pests. Residual levels are minimized by low application rates. Fenvalerate is most toxic to bees and fish. It is found in some emulsifiable concentrations. It is most commonly used to control insects of food feed and cotton products, and for the control of flies and ticks in barns and stables. Fenvalerate does not affect plants, but is active for extended period of time. Fenvalerate may irritate the skin and eyes on contact and it also harmful if swallowed (Fischel, 2005).

2.5.1.4. Carbamates

Carbamates are esters of carbamic acid. Carbamates are not structurally complex. Presently the volume of carbamates used exceeds that of organophosphates because they are considered to be safer than organophosphates. Carbamate pesticides are derived from carbamic acid. They are widely used in homes, gardens and agriculture. Like organophosphates, their mode of action is inhibition of cholinesterase enzymes affecting nerve impulse transmission.

Most of the carbamates are extremely toxic to Hymenoptera and precautions must be taken to avoid the foraging bees or parasitic wasps. Some carbamates are translocated within plants, making them an effective systematic treatment.

2.5.1.3. a. Carbaryl

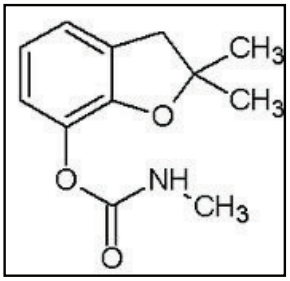


Carbaryl, the first carbamate introduced in 1956 and more of it has been used throughout the world than all other carbamates combined. Carbaryl, 1-naphthyl methyl carbamate, is a chemical in the carbamate family used chiefly as an insecticide. It is a white crystalline solid commonly sold under the brand name sevin, a trademark of the Bayer Company.

Carbamate insecticides are slowly reversible inhibitors of the enzyme acetylcholinesterase. The development of the carbamate insecticides has been called a major breakthrough in pesticides. The carbamates do not have the persistence of chlorinated pesticides. Although toxic to insects, carbaryl is detoxified and eliminated rapidly invertebrates. It is neither concentrated in fat nor secreted in the milk. For this reasons, carbaryl is favoured in food crops. Carbaryl kills both targeted and beneficial insects as well as crustaceans (Lahr *et al.,* 2000).

2.5.1.3. b. Carbofuran

Carbofuran is one of the most toxic carbamate pesticides. It is marketed under the trade names Furadan and Curater. It is used to control insects in a wide variety of field crops, including potatoes, corn and soya beans. It is a systemic insecticide, which means that the plant absorbs it through the roots, and from here the plant distributes it throughout its organs where insecticidal concentrations are attained. Carbofuran also has contact activity against pests.



Carbofuran is highly toxic to vertebrates particularly birds. It its granular form, a single grain will kill a bird. Carbofuran has been illegally used to intentionally poison wild life in the US, Canada and Great Britain. Secondary fatal poisoning of domestic and wild animals has been documented (Wobeser *et al.*, 2004) specifically bald and golden eagles, domestic dogs, raccoons, vultures and other scavengers. In Kenya, farmers are using carbofuran to kill lions and other predators (Mynott, 2008). Carbofuran has one of the highest acute toxicities to human of any insecticide widely used on field crops (Shetty *et al.,* 2011).

2.5.2.1. Toxicity tests

2.5.2.1.1. Stock solution

The stock solution of toxicant (1000 ppm) was prepared by dissolving 1ml of toxicant with 50 ml of water for proper dissolving and then the solution was made up to 1000 ml to obtain a stock solution. From this stock solution, appropriate quantity was taken as desired concentration.

2.5.2.1.2. Short term toxicity

After preliminary trials elaborate short term (12, 24, 48, 72 and 96h) toxicity studies were carried out in triplicates exposing the test animals to a wide range of pesticide concentrations. Complete immobilization or death was the end point.

2.5.2.1.2.1. Nauplii

For short term toxicity tests, 10 *Artemia* nauplii (8 ± 2h old) were placed in triplicate in various test media and subjected to test conditions for 4 days (96h). Tests were done with food addition mortality was noted for every 12h and 12, 24, 48, 72 and 96h LC50 were calculated.

2.5.2.1.2.2. Preadult

Active and healthy nauplii (6 ± 1 day old) were treated with various test media in triplicate for 4 days (96 h). They were fed with 1% glucose during the experiment and the mortality was recorded for every 12h upto 96 h and 12, 24, 48, 72 and 96h LC50 were calculated.

2.5.2.1.2.3. Adult

Ten *Artemia* adults (13 ± 1 day old) were exposed to various test media in triplicate for 4 days (96h). They were fed with 1% glucose during the test period and the end point for every 12 h was noted. From this data, 12, 24, 48, 72 and 96 h LC50 were calculated. The probit analysis method (Finney, 1971) was used to evaluate the acute toxicity test.

2.6. Statistical Analysis

The data obtained in the present study were subjected to the following.

1. Standard deviation.
2. Regression analysis.
3. One way Analysis of Variance.

2.6.1. Standard Deviation

$$SD = \sqrt{\Sigma d^2 / N - 1}$$

Where, d refers to the deviation of each score from mean and N is the total number of samples.

2.6.2. Regression analysis

Regression analysis was done with the help of regression models from the computer program SPSS 11.0 from windows 2001.

2.6.2.1. Regression equation

$$Y = a \pm bx$$

This expression would be recognized as a general equation for a straight line.

2.6.2.2. Regression coefficient

The parameter b is termed as the regression co-efficient or the slope of the best fit regression line.

2.6.2.3. Goodness of fit test.

The Chi square(x^2)goodness of fit test indicated whether the model sufficiently described the relationship between concentration and mortality where the calculated goodness of fit chi-square value was significant, a heterogeneity factor was used in

the calculation of confidence limits. The critical value at 5% probability was noted form the table of critical values of the Chi-square distribution (Zar, 1999).

2.6.2.4. LC50

The LC50 concentration was lethal to 50% of the test organism was observed and calculated with the help of regression models from the computer programme SPSS 11.0 for windows 2001. The calculations were done under probit model with mortality as response frequency and concentration as co-variate which was transformed to log base$_{10}$. The LC50 of 12, 24, 48, 72, and 96 hr nauplii, preadult and adult *Artemia* were calculated for organochlorine, organophosphoate, pyrethroid and carbamate.

2.6.2.5. 95% confidence (fiducial) limts

To define the limits of the calculated LC50 taking into account the variability of the system, 956% confidence limits called fiducial limits (upper and lower) were calculated with the help of the computer programme SPSS 11.0 for windows 2001.

2.6.2.6. fLC50

According to Reish and Oshida (1987)

Lower limit = LC50/ fLC50

Using this formula fLC50 was calculated.

2.6.3. One way Analysis of Variance

The effect of one factor on a variable was analyzed following the procedure described by Zar (1999). Values obtained for different groups were taken in different columns. For each Colum Σx and Σxwere calculated. Sum of Σx for all the columns

were squared and divided by the number of tabulated values and a correction factor C was obtained.

$$C = \frac{(\text{Sum of all } \Sigma x2)}{N}$$

Total SS (Sum of square = Sum of Σx^2 for all row scores) = C

$$\text{Between SS} = \frac{(x^2) \text{ of each column}}{\text{Number of values in each column}} - C$$

Error SS (Within SS) = Total SS – Between SS.

Considering the degrees of freedom of each source of variance, mean square (MS) was calculated.

Degrees of freedom DF

Total DF = Number of values on the table 1.

Between DF = Number of columns – 1.

Error DF = Total Df – Between DF

$$\text{Between MS} = \frac{\text{Between SS}}{\text{Between DF}}$$

$$\text{Error MS} = \frac{\text{Error SS}}{\text{Error DF}}$$

$$\text{F value for the variance between columns} = \frac{\text{Between SS}}{\text{Between DF}}$$

Significance level at the corresponding DF was read from the table.

2.7.1. Effect of sublethal concentrations

2.7.1.1. Rearing media for chronic toxicity studies

The desired concentrations of the pesticides were prepared by diluting the stock solution and then adding the required quantity, to bring it to the test concentration.

Lindane	-	0.002 µg/L – 0.04µg/L
Endosulfan	-	0.002 µg/L – 0.04µg/L
Malathion	-	0.0002 µg/L – 0.004µg/L
Monocrotofos	-	0.0001 µg/L – 0.002µg/L
Cypermethrin	-	0.00025 µg/L – 0.0005µg/L
Fenvalerate	-	0.00025 µg/L – 0.0005µg/L
Carbaryl	-	0.0025 µg/L – 0.005µg/L
Carbofuran	-	0.0025 µg/L – 0.005µg/L

2.7.1.2. Rearing

In order to study the effect of pesticides on the survival, growth and reproductive characteristics, ten larvae were introduced into 100 ml of the test medium within 10 hours of hatching and were fed with 1% glucose twice daily. The culture medium was changed once in four days. The salinity of the medium was checked every morning and evening. The volume and salinity of the medium were kept constant. Survival and growth were observed up to 30 days and the reproductive characteristics were observed till death of the female. The experiments were done in 3 or 5 replicates. A control group containing similar number of organisms was also maintained.

2.7.1.3. Survival

10 larvae were reared in 250 ml glass bowls containing 100 ml of the test medium (in 3 to 5 replicates) in ambient temperature (28 ± 2^{o}C). They were fed with

1% glucose twice daily and mortality observed every morning and evening for 30 days. To counter check, the number of survivors was counted every alternative day. For every five days, the average percentage of survival in each medium (control as well as experiment) was calculated and compared.

2.7.1.4. Growth

Growth in terms of length was measured for 30 days. Growth was determined by measuring the length of *Artemia* for anterior margin of the lead in from of the ocellus to the base of the caudal furca (Douillet, 1987). On every 5^{th} day, length of 5 randomly chosen larvae (in five replicates) was measured with the help of a dissection microscope, the platform of which had a graph paper glued to it. The average growth for every fifth day in the respective medium was calculated and compared.

2.7.1.5. Reproducive characteristics

After maturation, when the *Artemia* start riding, each pair was isolated and reared separately in 100 ml of the respective medium (with 3 or 5 replicates).Every morning and evening each pair was observed with hand lens to study the following characteristics.

1. Maturation period

This was counted from the day of hatching to the development of ovary.

2. Gestation period

It is the duration between the appearance of ovary and the first brood parturition.

3. Pre-reproductive period

This period is scored from the day of hatch to the first parturition day.

4. Reproductive period

The period extends from the first parturition day till the last parturition day.

5. Post reproductive period

It is the life time after the last parturition.

6. The number of larvae released

7. The number of cyst released

From the above observation, the following characteristics were calculated.

1. Total number of offspring / individual
2. Number of larvae / brood
3. Number of cysts / brood
4. Total number of offspring / brood
5. % of offspring encysted
6. Total brood / individual
7. Average interbrood duration.