EFFECT OF COW URINE DISTILLATE ON THE GROWTH, SURVIVAL AND IMMUNE SYSTEM OF *MESOCYCLOPS LEUCKARTI*

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IN

ZOOLOGY

By

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CERTIFICATE

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DECLARATION

The research work presented in this dissertation entitled "**EFFECT OF COW URINE DISTILLATE ON THE GROWTH, SURVIVAL AND IMMUNE SYSTEM OF *MESOCYCLOPS LEUCKARTI"*** has been carried out under the guidance of **Dr. S. VENKATALAKSHMI**, Head and Associate Professor, **Department of Zoology**, Government College for Women (Autonomous), Kumbakonam – 612 001. This work is original and has not been submitted in part or full for any degree or diploma of this or any other University.

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LIST OF ABBREVIATION

%	-	Percentage
±	-	Plus or minus
µl	-	Microliter
µm	-	Micrometer
‰	-	Parts per thousand
^{o}C	-	Degree Celsius
cm	-	Centimeter
DHA	-	Docosahexaenoic acid
EFA	-	Essential fatty acids
EPA	-	Eicosapentaenoic acid
Fig	-	Figures
g	-	Gram
$H_2 SO_4$	-	Sulphuric acid
HCl	-	Hydrochloric Acid
mg	-	Milligram
ml	-	Milliliter
mm	-	Millimeter
nm	-	Nanometer
mmol	-	milli mole
µmol	-	micro mole
U	-	Unit/Units
v/v	-	volume/volume
v/w	-	volume/weight
rpm	-	Rotate per minute
SE	-	Standard error
PBS	-	Phosphate Buffer Saline
N	-	Nauplii
PSU	-	Practical salinity unit
Org	-	Organism
pH	-	Hydrogen-ion Concentration
PUFA	-	Poly unsaturated fatty acids

HUFA	-	Highly unsaturated fatty acids
sp.	-	Species
DO	-	Dissolved Oxygen
COD	-	Chemical Oxygen Demand
BOD	-	Biological Oxygen Demand
TN	-	Total Nitrogen
IP	-	Inorganic Phosphate
PO_4^{3-}	-	Phosphate
NO_3^-	-	Nitrate
NO_2^-	-	Nitrite
NH_3	-	Ammonia
Temp	-	Temperature
SiO3	-	Silicate
CUD	-	Cow Urine Distillate
SA	-	*Staphylococcus aureus*
KP	-	*Klebsiella pneumonia*
EC	-	*Escherichia coli*
SF	-	*Shegella flexnieri*

INTRODUCTION

Aquaculture has shown impressive growth in the supply of fish for human consumption. Aquaculture provided only 7% of fish for human consumption in 1974, and was increased to 26% in 1994, 39% in 2004 and 45% in 2016 (FAO, 2018). Global aquaculture production (including aquatic plants) in 2016 was 110.2 million tonnes and in 2018 was 156 million tonnes, with the first-sale value estimated at USD 243.5 billion. Aquaculture is one of the major sources of providing food and income in several countries, which is also asians significant base for nutrition and livelihood of millions of people around the world (FAO, 2018). World per capita fish supply reached a new record of 20 kg in 2014, to vigorous growth in aquaculture, which now provides half of all fish for human consumption, and to a slight improvement in the state of certain fish stocks due to improved fisheries management. Moreover, fish continues to be one of the most-traded food commodities worldwide with more than half of fish exports by value originating in developing countries.

In India, fisheries is found to be a major economic activity that is flourishing with various resources and potentials. After the independence of the country, the aquaculture has been recognized as an important sector of agriculture. The growth of this sector can be identified clearly from the eleven-fold increase of production in last six decades as 0.75 million tonnes in 1950 to 1951, 9.6 million tonnes in 2012 to 2013 (FAO, 2014) and 10.07 million tonnes in 2014 to 2015 (Jothi Sivagnanam, 2016). This resulted in an unparalleled average annual growth rate of over 4.5 percent over the year which has placed the country on the forefront of global fish production, only after China (Hiralal jana, 2016).

India's aquaculture production basically can be classified into freshwater and brackish water production. The culture systems adopted in the country vary greatly

depending on the input available in any particular region as well as on the investment capabilities of the farmer. With the understanding of the biological basis of fish production, a series of systems are available with varying levels of inputs and outputs, and these can be categorized as low, medium and high input technologies. Low–input system: The natural productivity of this system is increased by using low-cost inputs such as organic and inorganic fertilizers, aquatic weeds etc. Medium - input system: In this system, supplementary feeding is provided apart from additional elements of fertilization for enhancing the fish production. High – input system: Higher stocking density combined with higher feed inputs is the typical characteristics of intensive culture system aimed at higher fish production from unit area. Such high input based culture system uses a balanced diet together with intensive aeration and water replenishment apart from other inputs as medicines, probiotics etc.

Tamil Nadu state is one of the leading states of India for fisheries development. The state is a pioneer in the country where the department of fisheries was made during 1907 itself. Fish culture in the state had received remarkable attention as early as in 1911, subsequently by other states of India. The fisheries in the state play a major role in food security with 2,500 different species in the different aquatic environment. Tamil Nadu fisheries department, having a second longest coastline in the country with abundant natural resources for coastal aquaculture. The state contributes 10 to 12 percent of the total marine fish production in the country and total estimated potential area for brackish water aquaculture is 14,880 hectors in which only 4,455 hectors are used for culture, it shows there is vast area readily available for brackish water aquaculture (Jayapal, 2014).

Aquaculture receives increasing importance in the country as it is a means for augmenting food production, an enterprise for improving the economy and an

operation for productive utilization abandoned land and water spreads. Resources of land, water and cultivable species of fish and prawn are available extensively in the different states of India. The success of aquaculture largely depends upon various management techniques which include a production of live food organisms so as to support the hatchery for the production of quality seeds.

The most important requirement of the aquaculture practice is a production of appropriate, nutritional, balanced, unpolluting, economically viable and readily acceptable feed in order to realize optimum growth and survival of the cultivable stock. Live food organisms are preferred by most of the cultured fish larvae compared to artificial supplementary feed. The zooplanktonic population forms the important major live feed organism in the natural environment. Aquatic ecosystem, where life originated, is the most diverse ecosystem in the world. Water is vital for the existence of all living organisms, and human civilizations are centered on it.

The quantity of freshwater on earth is very small in comparison to the waters of the ocean, but freshwaters have much more rapid renewal rates. Freshwater mainly is in the form of ice, snow and groundwater. Only 0.01% of the global freshwater is available in rivers, lakes and reservoirs. Inland waters cover less than 2% of the earth's surface, almost four fifths of which is contained in lakes and dam reservoirs. Streams and rivers within their drainage basins are central to the surface water ecosystems. Rivers constitute an insignificant amount (0.001 or 0.1%) of the land surface, and only 0.0001% of the earth's water occurs in river channels (Wetzel, 2001).

Freshwater habitats can be further divided into lentic and lotic ecosystems, based on the difference in the water residence time and the flow velocity (Odum, 1971). The water residence time in a lentic ecosystem on an average is 10 years and

that of the lotic ecosystem is 2 weeks. The surface waters sustain freshwater biodiversity, perform ecological functions and support human needs such as agriculture, hydro-electricity, industry, sewage and sanitation, aquaculture, fisheries, drinking water, transportation, recreation and spiritual needs. Freshwater organisms constitute about 25% of living organisms. The principal components of zooplankton in the lentic environment are represented by the taxonomic groups such as Protozoa, Rotifera, Cladocera, Ostracoda and Copepoda. Zooplankton incorporates primary and partly secondary micro faunal consumer operative systems (Zafar *et al.,* 2005). In an aquatic system, solar energy is the main source of energy that is trapped by the phytoplankton the producers, which in turn are consumed by the zooplankton, which are the primary consumers (Abbasi, 2005).

The first use of the term 'plankton' is attributed to the German marine biologist, Hensen in1887. In the latter half of the nineteenth century, he began a series of expeditions to explore the distribution, abundance and composition of microscopic organisms in the ocean (Battish, 1992). According to Hensen, 'plankton' (singular-plankter) includes all organic particles which float freely and involuntarily in the open water, independent of shores and bottom. In Greek, plankton means wandering. Hensen's plankton also included the non-living particles. Later, Wilhelmiin (1917) coined the term 'tripton' for non-living particulate matter and it is still valid. Plankton now includes living organisms and can be divided into phytoplankton and zooplankton. They can further be divided on the basis of size: megaplankton more than 8 cm (*Physalia, Vellela, Porpita*, all marine sp.), macroplankton-size vary from 1mm to 1cm, mesoplankton - 0.5 to 1mm, microplankton - 0.06 mm to 0.5 mm, nanoplankton - 0.005 mm to 0.06 mm, and ultraceston -0.0005 mm to 0.005mm. Nanoplankton normally passes through a tow net, while all the other categories of

plankton can be caught in tow nets and are also referred to as net plankton (Dussart and Defaye, 2001).

Plankton is a highly valuable food and plays an important role in the purification of polluted waters. Zooplankton plays a pivotal role in sustaining, especially, young fishes, it also forms much of the food for planktivorous fishes and also supplements food of other fishes. Its importance to practical aquaculture cannot be overstated (Alekseev, 2002).

Zooplankton is frequently used in the ecotoxicological investigation because they are one of the groups most sensitive to toxic chemicals and they occupy a central position in the lentic (standing water) food chain. The responses of zooplankton to toxicity tests are considered to be informative of relative impacts on the ecosystem as a whole (Hanazato, 2000). Crustacean zooplankton is an important component of marine and freshwater food webs since they are the primary grazers in many ecosystems and are often the major food source for developing larvae and fish. Contaminant impacts on these animals are of interest since they can affect food web structure by altering the grazing on phytoplankton communities and by affecting the food supply of predators (Sharon and Nicholas, 2000).

Zooplankton occupies a central position between the autotrophs and other heterotrophs maintaining an important link in the sustainability of the food chain forming one of the most important components of freshwater aquaculture species (Chakrabarti and Sharma, 1998). In semi-intensive or intensive culture conditions, aquaculture species derive a substantial part of their dietary nutrient needs from naturally available zooplankton as they are a valuable source of protein, amino acids, lipid, fatty acids, vitamins and enzymes (Millamena *et al.,* 1990; Munilla-Moran *et al.,*1990; Pillay, 1990; Evejemo *et al.,* 2001). Although in semi-intensive fish culture,

the cultured species draws a significant part of nourishment from zooplankton grown in ponds, quantization reveals its nutritional contribution to fish growth is limited. However, the dependence on live food larvae makes it pertinent to evaluate the nutritional composition of live food in aquaculture (Srivastava *et al.,* 2006).

Copepods, one of the major components of the zooplankton group are very ancient arthropods and the diminutive relatives of crabs and shrimps (Reddy, 2001a). These minute crustaceans lack a distinct shell fold and have a simple median eye. In their free-living forms, the body is elongated and segmented. Copepods are claimed to be numerically the most abundant metazoans on earth and conservative estimations revealed that they likely outnumbered the abundance of insects (Williamson and Reid, 2001). In terms of their size, diversity and abundance, they are often called 'water fleas' in common with many other small crustaceans (Reddy, 2001b). They fossilize poorly, and thus it is rare to find traces of their remains in sediments, which could have facilitated the study of their morphological, physiological and ecological evolution (Frey, 1964). Copepods are associated with the aquatic environment, but can also tolerate low humidity. In the oceans, they are most successful in pelagic waters, but can also inhabit the sediments. In freshwaters too, they are present in many different biotopes, as well as in the humid litter of deciduous forests, in decaying leaf- mold of hollow trees, inside ant nests etc (Armengol and Miracle, 1999). Zoogeographical data suggest that there was a rich, diverse fauna of copepods by the tertiary period and that different species had much time to adapt to the conditions of the environment (Dussart and Defaye, 2001).

Copepods occur in almost all freshwater habitats. They are present in large ancient lakes, pools of glacial meltwater, hot springs, hypersaline lakes, ponds, rivers and subterranean waters. With an estimated 13,000 taxonomic species known, the

greatest diversity of copepods is found in the marine environment, but approximately 2,814 species under 257 genera inhabit freshwater (Boxshall and Defaye, 2008). Huys and Boxshall (1991) hypothesized that copepods originated in the marine environment, and all ten orders of Copepoda had their origins in the marine hyper benthic community. They suggest that retreating glaciers may have aided the marine forms reaching coastal freshwaters and from there through flood waters and dispersion of diapausing eggs reached inland waters, whereon they diversified. Most freshwater copepods are free-living, but some are parasitic, mostly on fish hosts. Some live as commensal epibionts on freshwater invertebrates.

Boxshall and Defaye (2008) in their study on the global diversity of freshwater copepods state that the Palaearctic realm exhibits the highest diversity in freshwater copepods with approximately 1,204 species belonging to 134 genera of which 35 genera are endemic to the realm. The major contributors to diversity are the Cyclopidae. The Neotropical region has the second-highest diversity, with 561 species, the major contributors again being the Cyclopidae (31%). However, the highest endemicity was exhibited by the Neotropical region with 54 endemic genera out of the 104 genera represented there. The least diversity is observed in the Antarctic region with 17 species belonging to 14 genera, with no endemics. The oriental region has a relatively low diversity, represented by 381 species belonging to 79 genera and 16 families. 19 genera are endemic to the area. The major contributors are the Cyclopidae (30%), especially *Mesocyclops* and *Thermocyclops*, the Diaptomidae (24%), mostly *Tropodiaptomus* and *Heliodiaptomus*, the Canthocamptidae (15%), mainly Elaphoidella, and the Lernaeidae (12%). Members of the families Cyclopidae in the Cyclopoida and Diaptomidae in the Calanoida are

highly successful in all kinds of freshwater habitats, and mostly represent the Indian planktonic Copepoda (Reddy, 2001b).

Copepods play an important role in ecosystems, by virtue of their place in the food webs as primary and secondary consumers, as well as by their potential to be used by man in different ways (Dussart and Defaye, 2001). Their grazing contributes to the transfer of algal primary production to higher tropic levels i.e., they make organic material available to higher tropic levels in larger pellet form, thus saving the foraging energy of their predators (Reddy, 2001b). Cyclopoids such as *Microcyclops, Megacyclops* and *Mesocyclops* can be used as biological agents in mosquito control, (Marten *et al.,* 1994; Marten and Reid, 2007) and *Paracyclops* to control plant-parasitic nematodes (Reversat G, 1992). Copepods find application in monitoring studies in the field of functional genetic and transcriptomic studies and laboratory. The calanoid/cyclopoid-cladoceran ratio is used in limnological studies as a water quality indicator. Cyclopoids, mostly *Mesocyclops* spp., act as vectors of human parasites, of which the most important is the guinea worm, *Dracunculus medinensis*. They are also known as significant chitin producers in planktonic and benthic ecosystems (Reddy, 2001b).

The freshwater zooplankton of tropical regions is less diverse than that of temperate regions (Fernando, 2002). However, the reasons for such a difference are not yet clear. Fernando opined that direct and indirect effect of temperature, the type of food available, predation by fish and invertebrates, toxic effects of algae and macrophytes all influence species composition in the tropical region. In general, the uniform temperatures existing throughout the year in the tropics would not favour diversity of zooplankton species in. It is possible that zooplankton production is low compared to that of temperate lakes. Limnetic cyclopoid copepod species in any

tropical region is restricted to two or three species; maybe due to their ability to feed on blue-green algae or carnivores. The evenly high temperatures may reduce species diversity and eliminate cold water species in general.

The Cyclopoid Copepoda is among the most ubiquitous microcrustaceans in lentic freshwaters, yet their occurrence in the tropics is poorly documented (Fernando and Ponyi, 1981). Tropical Cyclopoida in freshwaters is poor in species compared to sub-tropical and temperate regions. Some of the earlier works on freshwater Cyclopoida of humid tropical Asia include systematic studies from Burma (Lindberg, 1949); Cambodia (Kampuchea) (Lindberg, 1941); India (Kiefer, 1939; Lindberg, 1952) and Indonesia (Kiefer, 1933). The systemic works are by Fernando (1974) and Fernando and Ponyi (1981) on Sri Lankan Cyclopoids. Ecological factors affecting copepod species from western ghats of Maharastra were reported by Kulkarni, 2016 and new copepod species were identified from lakes of Karnataka (Jayasree loka, 2017) and in Tamil Nadu (Manickam *et.al,* 2012; Kalpana *et al.,* 2017).

Scientific reports on Indian freshwater copepods began to appear with the works of Gurney (1906, 1907 and 1930), who gave an account of Indian Diaptomids, including the description of four new species from Calcutta and Chota Nagpur from collections in the Indian Museum. The last forty years have seen much-published work on freshwater zooplankton, especially freshwater copepods of temperate countries, but still, a comprehensive work in this field is required. Knowledge of the freshwater copepods of India, and on factors influencing their diversity, ecology and population seasonal dynamics are very scanty. In tropical countries like India macroorganisms like fishes, crustaceans and mollusks have been studied in depth and our knowledge is comparatively better. But, the microorganism especially the phytoplankton and zooplankton have not been studied detail in Tamilnadu freshwater

ecosystem. Therefore, the present study was to investigate the population and Seasonal dynamics of free-living freshwater zooplankton of three local ponds in Kumbakonam, Thanjavur District, Tamilnadu.

In recent years, with the rapid development of the aquaculture enterprises, the infectious diseases caused by viruses, bacteria and parasites became more severe, resulting in huge economic losses. At present, information on the immune status of the microcrustaceans in copepods is scarce (Sahoo *et al.,* 2005). Hence in the present study an attempt was made to explore the immune system in the selected freshwater copepod.

Aquaculture industry faces two main challenges. One is the feed economics cost benefit analysis and the other being diseases. Microorganisms like bacteria, viruses, protozoans and fungi are mainly responsible for the increased infections in fishes. These organisms possess the genetic ability to gain resistance towards synthetic chemotherapeutic drugs, and on the contrary, the host experiences many adverse effects of these drugs. They also affect the non target organisms like the beneficial planktons which are the back bone of food chain in freshwater ecosystem. Hence to rectify such abnormal and grave situation, researchers are focusing on finding naturally available products. Nature is an abundant first rate store-house consisting of an enormous range of plants, animals and microorganisms available for the discovery and further development and supply of these products, which are capable to counter almost all microbial infections prevalent in humans and animals. The Indian Cow, *Bos indicus*, is a most sacred and valuable animal in religious scriptures. Cow urine has found therapeutic applications since days of the year. Cow urine is consumed by the majority of the rural population as a traditional remedy almost in the whole Indian continent (Jerald and Edwin, 2008).

Cow urine has a great pharmacological importance Its medicinal utility has been greatly mentioned in depth in Ayurveda. Cow urine is found to be effective against reversal of the certain cardiac and kidney diseases, indigestion, stomach ache, edema, skin disease etc. (Arunkumar *et al.,* 2010). The cow urine distillate has been patented as bio activity enhancer and availability facilitator for biomolecules including anti- infective and anticancer agents (US Patent No 6410 059/2002). Cow urine has certain volatile and non-volatile components which might have high antimicrobial activity (Shaw *et al.,* 2007).

Further, no such effort has been carried out to see the immunological aspects of freshwater zooplankton (copepod) in Indian freshwater ecosystem, particularly to understand how different degrees of parasitic problem influence the survival of host by modulating the innate immune mechanism. Bhujel *et al.,* (2010) reported the use of cow urine of as continuous pond fertilizer on a weekly basis as a splitting doze in Raina's Tar of Lamjung district in mid-hills of Nepal. A two year's project conducted to improve the empowerment of women through small-scale aquaculture used cow urine and proved it is as one of the suitable nutrients for the growth of fish. Cow feces and urine are beneficial to sieving fish and omnivorous fishes (Kumar and Ayyappan, 1998). The collection of nitrogen-rich urine as a pond input has significant potential (Edwards *et al.,* 1988). Cow urine not only provides nutrition for fish but also helps for the growth of planktons in the ponds (Radheyshyam *et al.,* 2012). Yet other researches also revealed the immunomodulatory properties of cow urine (Nair, 2002; Bhadauria, 2002; Banga *et al.,* 2005; Chauhan, 2004; Chauhan *et al.,* 2009; Chawla *et al.,* 2010; Tiwari, 2015 and Sunil Kumar *et al.,* 2017). Hence, an attempt has been made to study the cow urine distillate as working immune modulator or immune stimulator for freshwater Zooplankton.

SCOPE AND OBJECTIVES

India is the third largest aquaculture producer in the world, which has a coast line of over 8129 km^2 including Andaman and Nicobar and Lakshadweep islands with 2.02 million km^2 EEZ. But the contribution in freshwater aquaculture is very meager. This is due to the failure in seed production technology for fin and shell fishes. It is believed that failure in seed production of fish was might be due to the lack of suitable nutritionally balanced live feeds during the early stages of larvae. Freshwater aquaculture systems ranging from intensive pond- or cage-based systems to extensive stocking of enclosed water bodies play an important role for the nutrition and livelihood of rural people. Often, these systems are integrated with some form of agriculture, making use of farm by-products. Several earlier workers confirmed that copepods are promising alternative live feed in hatchery. Hence the present study focused on culture of copepods. The present study was attempted with the following specific objectives:

1. To study the zooplankton diversity and richness of the selected ponds.
2. To study the seasonal dynamics of zooplankton population.
3. To correlate between water quality and zooplankton diversity.
4. To Identify the dominant zooplankton to consider as the candidate species for live feed culture.
5. To study different components of immune system in the selected copepod.
6. To study the influence of cow urine on the growth, survival and immune system of copepods.

Plankton (phyto and zoo) is an essential ecosystem factor which responds to environmental alterations rapidly. This effect is attaining due to key role of planktonic organisms in the turnover of organic matter and energy through the ecosystem.

Plankton studies in strained ecosystems are generally intended at achieving two main objectives. First, based on primary knowledge on plankton species composition, density, and physiological state of organisms, it is possible to evaluate the level of water pollution. Secondly, it is vital to know the probability and feasible rate of water ‘‘self-purification’’ due to filtering and metabolic activities of planktonic organisms. The analysis of Zooplankton dynamics was used by various researchers for inferring the environmental conditions of the water bodies, for assessing the trophic status of the ecosystem (Frank *et al.,* 2006) and to understand the factors regulating population composition and abundance. Hence a thorough literature review was done for the present investigation for the proper understanding of the research problem, exact need for the society and to identify the literature gap.

1. Freshwater Zooplankton

Freshwater Zooplankton are heterogeneous assemblage of free-floating microscopic animals. Zooplankton of freshwater ecosystem are comprised various invertebrate group and the major groups are rotifera, cladocera, and copepoda.

Rotifera

The name "rotifer" is derived from the Latin word meaning "wheel-bearer"; this makes reference to the crown of cilia around the mouth of the rotifer. The rapid movement of the cilia in some species makes them appear to whirl like a wheel.

Rotifers are microscopic aquatic animals of the phylum Rotifera. Rotifers can be found in many freshwater environments and in moist soil, where they inhabit the thin films of water that are formed around soil particles. The habitat of rotifers may include still water environments, such as lake bottoms, as well as flowing water environments, such as rivers or streams. Rotifers are also commonly found on mosses and lichens growing on tree trunks and rocks, in rain gutters and puddles, in soil or leaf litter, on mushrooms growing near dead trees, in tanks of sewage treatment plants, and even on freshwater crustaceans and aquatic insect larvae. (Orstan, 1999).

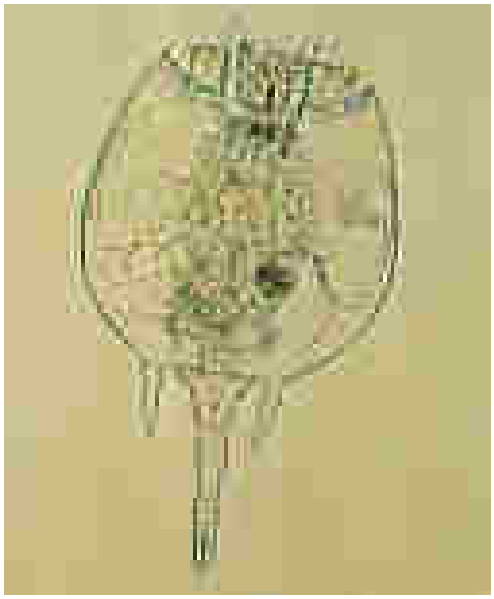


Plate 1. Rotifera groups

Cladocera

Cladocerans are small crustaceans commonly found in most freshwater habitats, including lakes, ponds, streams and rivers. While there have been a few marine species, cladocerans have definitely not been successful in seawater. Bodies of freshwater that lack an abundance of fish that act as predators provide the most suitable habitats. Many species of cladocerans can be found residing in the open water of lakes, as do plankton. Some other species live either on or near vegetation near the

bottom of lakes. Generally, cladocerans are in motion most of the time, swimming by vigorously stroking their antennae, which are used as their main form of propulsion. Most species of cladocerans also move in a series of hops, in the same manner as fleas, hence their nickname "water fleas" (Fryer 1993). In addition to swimming and hopping, many cladocerans spend a large amount of time crawling in mud, leaf surfaces, or other bottom debris. They accomplish this crawling motion by kicking their legs, which also resembles hopping (Pennak 1978).

Plate 2. Cladocera groups

Copepoda

Copepods are a group of generally minute aquatic crustaceans found in marine waters and nearly every freshwater habitat. Copepods have a segmented, bullet-shaped body. The head is fused with the first one or two thoracic segments. Thorax is cylindrical, followed by narrower abdomen. Abdomen lack appendages, except for two spiny tails (rami). Copepods have a single (mostly reddish) spot eye. Adult female copepods of the order Calanoida carry single bundle of eggs attached to their abdomens. Adult female copepods of the order Cyclopoida have parried bundles of

eggs. These micro crustaceans occupy the major Zooplankton community in the selected ponds of the present study. Hence their classification is seen further out of interest.

Taxanomy of Copepod

Phylum	: Arthropoda
Class	: Crustacea
Sub- Class	: Copepoda (Milne Edwards, 1840)
Super Order	: Gymnoplea
Order	: Platycopioida
	Calanoida
	Misophrioida
	Cyclopoida
	Gelyelloida
	Mormonilloida
	Harpacticoida
	Poecilostomatoida
	Siphonostomatoida
	Monstrilloida

Among these the Cyclopoida and Calanoida are found to be the major groups in the selected ponds of the present study.

Calanoida

Plate 3. Copepoda groups

Calanoids often live in open water and by the surface, where they form the part of zooplankton. Characterized by long secondary antennae, and in the females, a single egg-sac. Includes Diaptomus. (Sharma and Michael, 1987; Murugan *et al.,* 1998; Altaff, 2004).

Cyclopoida

Cyclopoids mostly inhabit the upper layer of sediments on the bottom, as the members of benthos community. Includes the well-known Cyclops, of which there are over a hundred species. Moderately long secondary antennae, and the females carry twin egg-sacs (Sharma and Michael, 1987; Murugan *et al.,* 1998; Altaff, 2004).

Plate 4. Cyclopoid groups

2. Studies on Physicochemical parameters on Zooplankton diversity

Zooplankton diversity and Physico-chemical conditions in three perennial ponds of Virudhunagae district, Tamilnadu were studied by rajagopal *et al.,* (2010) There is significant variation in the levels of Turbidity, pH, Total hardness, Phosphate, Salinity, Chloride and DO with respect to the three seasons was observed. The study indicated that water in Pennar river is highly contaminated and not safe for drinking. Poor quality of drinking water was recorded as the major risk factor for the

large-scale water-borne diseases in the area and negative relationship was found between water parameters and zooplankton diversity.

Joseph *et al.,* (2011) recorded the monthly changes in the abundance and biomass of zooplankton and water quality parameters in kukkarahalli lake of Mysore, India. About 53% of the variation in the abundance of Cladoceran, 55% of variation in the Cyclopoid copepod, 39% variation in the ostracod and 53% of variation in the abundance of total zooplankton were mainly due to pH. Negative relationship was found between the total zooplankton and concentration of phosphate as such as 67% decrease in wet biomass was mainly because of phosphate, where as 47% of dry biomass of total zooplankton was positively correlated with conductivity.

Sharma and Sharma (2011) studied the zooplankton diversity of loktak lake, Manipur, India. The limited influence of individual abiotic factors on zooplankton, with richness showing a significant inverse correlation with water hardness and chloride, and abundance inversely correlated with nitrate. Multiple regressions indicated higher cumulative effects of 15 abiotic factors on richness and abundance.

Goswami and Mankodi (2012) studied the zooplankton of freshwater reservoir nyari at Rajkot district in Gujrat. The range of zooplankton between 174 to 769 n/l, and average was 378.42 n/l, the minimum zooplankton was in March and maximum were in the month of October. Plankton density was high that coincides with the similar condition for nutrients as well as some physico-chemical property of water.

Amsha devi *et al.,* (2013) studied the physico-chemical parameters and zooplankton diversity of a temple pond in Virudhunagar, Tamilnadu. Totally 17 species of zooplankton were identified which includes 10 species of Rotifera, 3 species of Copepoda, 3 species of Cladocera and 1 species of Ostracoda. The seasonal

variations in water quality parameters of the pond have a marked influence on the numerical abundance of zooplankton.

Varadharajan and Soundarapandian (2013) studied the Zooplankton abundance and diversity from Pointcalimere to Manamelkudi, South East Coast of India. Overall, 73 species of zooplankton were noticed. In that 30 copepod species were recorded. The most dominant copepod species were *Acartia* sp., *Paracalanus* sp. and *Temora turbinate*. During the study period maximum salinity was recorded in the month of May 34.5 ppt. The distribution of some *Acartia* species is known to be affected by temperature and salinity regimes. Further the important factors that controlled the distribution of copepods were rainfall, river discharge and salinity as opined.

Suganthi *et al.,* (2014) analyzed the zooplankton diversity in certain local ponds of Cuddalore district, Tamilanadu, India. The identified zooplankton were, Rotifers comprises 9 species (36%), Cladocera -6 (24%), Copepoda -7 (28%) and Ostrocoda -3 (12%). The zooplankton diversity analysis shows an average abundance in winter and maximum occurrence in summer due to the different environmental characteristics of the fresh water body.

Manickam *et al.,* (2014) investigated the physico-chemical characteristics and zooplankton diversity from Perennial Reservoir at Thoppaiyar, Dharmapuri district, Tamil Nadu. Total of 55 species of zooplankton were recorded, which includes 19 species of Rotifera, 13 species of Cladocera, 15 species of Copepoda and 8 species of Ostracoda. Copepod was representated by 3 families 10 genera and 15 species. The copepod density was found to be maximum (1426 ind. /l.) in May and minimum (598 ind. /l.) in October. This study showed that the Rotifera species *Brachionus angularis*, *B. calyciflorus*, *B. falcatus* and *Filinia longiseta* and Cladocera sp. *Diaphanasoma*

sarsi, *Ceriodaphnia cornuta* exposed that the reservoir is being eutrophicated and polluted.

Manickam *et al.,* (2015) studied biodiversity of freshwater zooplankton community and physico-chemical parameters of Barur lake, Krishnagiri district, Tamilnadu. A total of 47 zooplankton species were identified which include 18 species of Rotifera, 11 species of Cladocera, 11 species of Copepods and 7 species of Ostracoda. It was shown that the distribution and diversity of zooplankton is depending on the physico-chemical parameters prevailing in the environment.

Sivakami *et al.,* (2015) investigated the density of zooplankton in a lake, Pudukkottai district, Tamilnadu, India. Totally 40 species were identified which includes two each belonged to Protozoa and Ostracoda, 27 to Rotifer, 5 to Cladocera, 3 to Copepoda and 1 to Anostraca. Seasonal changes in the pattern of zooplankton community have been driven by a combination of abiotic and biotic factors. Rotifer occurring highest numbers because of the rotifer's positive correlation with phosphate, dissolved oxygen and primary productivity.

Kather bee *et al.,* (2015) studied on plankton diversity and water quality of Ambattur lake, Tamilnadu. A total of 22 species of plankton consisting phytoplankton and zooplankton were recorded and found that the fluctuations among physico-chemical parameters influenced the diversity of plankton.

Shikha Panwa *et al.,* (2015) carried out studies on the diversity and distribution pattern of zooplanktons in the Bhimtal Lake, India. Totally, 29 species of zooplankton including 16 species of Rotifers, 8 species of Cladocera and 5 species of Copepod were observed. Positive co-relation was found between zooplankton along abiotic factors (pH and Temperature) and the zooplankton has adversely affected with

increasing alkalinity, nitrates and dissolved oxygen. The highest diversity of zooplankton was observed during summer season and lowest during winter. This lake was changed due to mesotrophic condition.

Diversity and seasonal variation of Zooplankton from Majalgaon reservoir in Maharashtra, India analysed by Rajkumar and Pawar (2016). Total of 23 species were found in this reservoir. Among these, rotifers comprised of 8 species (28.92%), Cladocera 6 (19.638%), Copepods 5 (20.09%), Ostracoda 2 (19.317 %) and Protozoa 2 (12.02). Copepods showed higher population density in summer season (927 org/lit) and lower in winter (688.666 org/lit). Abundance of copepods in summer and monsoon is due to the lake which is rich in organic matter supporting higher number of Cyclopoids. This study revealed temperature has important role in the distribution of zooplanktons in a fresh water habitat.

Rahul Pralhad Rathod *et al.,* (2016) investigated the Diversity Indices and Seasonal Variations of zooplankton in Kadwai Reservoir, Maharashtra, India. It was interpreted that owing to inflow of water and less photosynthetic activity by primary producers the abundance of zooplankton was observed very low in monsoon season and highest during postmonsoon season and zooplankton was showed maximum occurrence during postmonsoon season due to the presence of stocking fish seed.

Hedayati *et al.,* (2017) reported that the seasonal variations have influence on abundance and diversity of copepods in Mond River estuary, Bushehr, Persian Gulf. Totally four seasons from spring 2012 to winter 2013 were studied and reported 24 species of copepods belonging to 4 orders, 13 families and 10 genera. Among the 4 orders the Calanoida was most abundant (15 species) followed by Cyclopoida, Poecilostomatoida and Harpacticoida (3 species each). Moreover, the positive

correlation between the abundance of copepods at different seasons and physicochemical parameter such as salinity, pH, and temperature were observed.

Dhanasekaran *et al.,* (2017) studied the relation between water quality and Zooplankton diversity from Dharmapuri Lake, Tamil Nadu. Water quality parameters except DO (6.89±0.69), Temperature (26.51±0.95), pH (7.11±0.82), Salinity (1.206±0.532), TDS (141.50±30.65) and Electrical conductivity (1.378±0.589) showed significant seasonal variation. The highest values of the parameters occur between March to May (summer) and lowest between September to November (Monsoon). In addition, total Zooplankton (Individual/litre) were significantly more during the summer season than the winter. A total of 29 zooplankton species were identified qualitatively. The zooplankton was positively correlated with water quality parameters.

Seasonal variation in the diversity and distribution of zooplankton at Thandava reservoir, Visakhapatnam, India was studied by Rama Rao (2017). 44 number of Zooplankton species both micro and macro was observed in this reservoir. Between eight zooplankton groups, the diversity of Rotifera comprises of 17 species, Cladocera 8, Copepoda 5, Ostracoda 2, Protozoa 3, Crustacea 7, Fish larvae and Eggs 2. High number of copepods was observed in summer and low in the winter season. It was opined that the physico-chemical parameters and zooplankton communities collectively form a complete ecosystem and there is communication between the zooplankton and phytoplankton.

Diversity of Zooplankton from Chhapakaiya Pond Birgunj, Nepal was studied by Lal Babu Prasad Yadav *et al.,* (2017). 27 species of zooplankton belonging to three taxonomic groups were noticed in the pond. Maximum number of zooplankton was observed during summer and minimum during winter season. During this

investigation, copepods were constituted by 3 species namely Cyclops sp. Gammarus sp. and Nauplius larvae. Nauplius larva showed the maximum density within the member of Copepoda nearly in all the seasons. Cyclops and Nauplius were sensitive to pollution and increase with an increase in nutrients.

3. Studies on Copepod diversity

Eduardo Suarez-Morales *et al.,* (2004) carried out studies on the distribution of the freshwater cyclopine copepod fauna of the Yucatan Peninsula (YP), Mexico and its relationship with the geological and climatic history of this Neotropical karstic zone. Cyclopine colonization of the YP showed the influence of the South American fauna, as the closest relatives of some species endemic to the YP are South American forms; the Nearctic influence is low. The cyclopine fauna of the YP is formed by a mixture of Nearctic-derived (species of Acanthocyclops), Neotropical (i.e. *M. edax*, *M. longisetus*, *A. panamensis*, *Thermocyclops inversus* and *T. tenuis*), and epigean and hypogean endemic forms. The authors found that YP is a peculiar subregion that harbours a diverse fauna of cyclopine copepods with a high endemism.

Distributional patterns and diversity of the diaptomid calanoid copepods were analyzed by Suarez-Morales *et al.,* (2005) from Mexico and Central America. *Leptodiaptomus* and *Mastigodiaptomus* were found in the Neotrophical region. Mexico shares 33% of its species with North Americ and no species are shared between North America and South America. For the Diaptomidae, it was recorded that the Nearctic power was strongest in Mexico, elevated diversity present in South America, its influence in Mexico and Central America appeard to be Weak and neotropical diaptomid copepod fauna reflected the power of several dispersion trends.

Rajalakshmi *et al.,* (2010) examined the qualitative and quantitative analysis of copepod from Sodalaipuri estuary, Puducherry. 13 species of copepods were

recorded during the study period. Abundance of copepods was lowest during monsoon season, when the water column was clearly stratified to a large coverage because of high rainfall. Many copepod species disappeared during monsoon and species composition also changed, since they are mostly stenohaline. All the species were observed during summer followed by pre-monsoon and post-monsoon seasons.

Diversity and abundance of copepod from Wular lake, Kashmir Himalaya was studied by Javaid Ahmad Shah (2013). 16 species belonging to 3 families namely cyclopoids with 12 species, calanoids and harpacticoids being represented by two species each were recorded. The dominant species seen were *Cyclops bicolor*, *Eucyclops agilis*, *Bryocamptus nivalis* and *Diaptomus virginiensis*. At site III copepods were found to be dominant which were densely infested by macrophytes. The copepod species had more or less positive relations among themselves.

Debashri Mondal *et al.,* (2013) analyzed the diversity of cladoceran and copepods from Mirik Lake, Darjeeling Himalaya, West Bengal. The environmental parameters, mainly the water temperature, exert significant impact on the relative abundance of cladocerans and copepods. In the present study, maximum cladoceran density was observed at a temperature range of 9.00- 25.00 while the highest copepod density was recorded at a temperature range of 10 - 26^{o} C. The maximum densities of copepods were recorded in the pH range of 6.70-8.10 and 6.4-7.5.

Copepod diversity in southern European groundwater at different spatial scales was examined by Galassi *et al.,* (2013). 94 stygobiotic species were identified from this region. This study demonstrated the scale effect on the relationship between local and regional diversity by dissecting γ-diversity into α- and β-components. It showed the α-diversity were more controversial, suggesting that local assemblage saturation is scale-dependent and a reflection of local habitat structure and

evolutionary processes. The mean α- and γ-diversity of aquifers were not linearly associated and it represets a potential community saturation effect.

Diversity and abundance of planktonic copepods were analysed from Merambong Seagrass Meadow, Johor, and Peninsular Malaysia by Hazel Monica Matias-Peralta and Yusoff (2015). Overall, 48 species from 20 genera and 15 families were identified. Oithona spp and Paracalanus spp were very common in the seagrass area. Calanoids were the most abundant inhabiting all the stations, contributing 51.2% of the total copepod populations. This study showed the copepod diversity and richness were high and species evenness was relatively constant (0.9).

New records of an invasive calanoid copepod, *Arctodiaptomus dorsalis* (Marsh, 1907) in freshwater ecosystems in the Bicol Peninsula (Luzon Is., Philipine) were investigated by Rizo *et al.,* (2015). *A. dorsalis* was observed in Bicol River which is linked to Lake Buhi. This may be owing to an algal bloom (In Lake Bato) and low water level (In Lake Baao). This might point out that tremendously unfavorable environmental conditions may force *A. dorsalis* Populations to decrease or disappear altogether. Ability of *A. dorsalis* to generate resting eggs may help it to re found viable populations once conditions become better. *A. dorsalis* species was observed because of the cool dry season when algal blooms are less frequent and lake water levels are higher due to the just concluded southwest monsoon at the time.

Kulkarni and Kalpana Pai (2016) investigated the diversity and Species richness of freshwater Calanoid copepods from Western Ghats of Maharashtra, India. Totally, they found eleven species of diaptomid copepods. In that many of these are new records for this region. *E. shihi, H. kolleruensis, N. intermedius* and *M. pseudohebes* are known to be rare and were before thought to be limited only to

certain areas of India. Assemblage of constrained diaptomid copepods are identified in certain sites.

Hedayati *et al.,* (2017) investigated the abundance and biodiversity of copepods from Mond River (MR) estuary, Bushehr, Persian Gulf. Copepod assemblages were comprised of 4 orders, 13 families and 10 genera. The Highest number of diversity and distribution of copepods were observed in all seasons. They noticed the significant correlation between copepod diversity and abundance with physico chemical parameters such as salinity, pH and temperature. This study showed salinity factor was more effective environmental factor for copepod abundance.

4. Studies on Species Identification of Zooplankton

A DNA barcode database allows rapid and accurate identification of specimens and characterization of patterns of species distribution and diversity. Ann Bucklin *et al.,* (2009) analyzed the DNA barcoding of Arctic Ocean holozooplankton for species identification and recognition. Approximately 700base-pair region of the mitochondrial cytochrome oxidase I (mtCOI) gene was amplified and sequenced for 82 identified specimens of 41species. The mtCOI barcodes accurately discriminated and identified known species of 10 taxonomic groups of Arctic Ocean holozooplankton.

Jagadeesan *et al.,* (2009) analyzed the molecular discrimination of marine calanoid copepod *Paracalanus parvus* (Claus 1863) Using RFLP in Vellar Estuary, Tamil Nadu. RFLP stand for a stretch of DNA that serves as a marker for mapping a specified gene. In this study RFLP can be used to clearly distinguish the Calanoid copepod Paracalanus parvus from their closely related species and produced their own finger prints of 856bp, 691bp, 499bp, 285bp and 090bp.

Assessment of DNA integrity in freshwater zooplankton affected by pollutants from water treatment facility from croatia was investigated by *Ternjej et al.,* (2009). The level of DNA damage was observed during all the seasons. Calanoida, Daphnia and Chaoborus had the higher values of DNA damage in autum and spring at the polluted lake. Among this Cladoceran has highest genotoxic response of all zooplankton taxa studied. In this study showed the aluminium, when present in excess concentrations, to induce DNA damage in the cells of zooplankton organisms.

Combined molecular and morphological data of the freshwater copepod Mesocyclops from USA were investigated by Wyngaard *et al.,* (2010). Morphology includes polymorphic characters analyzed using this 18S rDNA and ITS2. *Mesocyclops darwini* was the single taxon whose relationships showed divergence among the previous reconstructions using only morphological characters and the tree inferred from the combined data setmolecules for phylogenetic analyses of copepods is such that morphology can contribute substantial information toward constructing topologies, as well as provide guidance in designing molecular studies.

Ho Young Soh *et al.,* (2012) reported the new species of *Pseudodiaptomus* from Korea using molecular data. Ribosomal internal transcribed spacer 1 and mitochondrial gene cytochrome oxidase subunit I sequences supported for this study. mtCOI and ITS I is an effective tool for discriminating the closely related species.

The patterns of morphological and molecular evolution from freshwater copepods in Italy was comparatively studied by Federico Marrone *et al.,* (2012). Currently three species were distinguished under the binomen *Hemidiaptomus (Occidodiaptomus) ingens* and two highly divergent clades within *H. (O.) roubaui*. This study showed a clear discordance among the molecular distances recorded,

Occido diaptomus morphological homogeneity, and prominence a remarkable decoupling among the morphological and molecular diversity in the subgenus.

Comparison of molecular species identification for North Sea calanoid copepods using proteome fingerprints and DNA sequences were investigated by Laakmann *et al.,* (2013) from Germany. 333 individuals of 13 calanoid copepod species were distinguished using COI and 18S rDNA and proteome fingerprints. In this study they observed species-specific protein mass spectra, underlining the power of this instrument in the application on metazoan species identification. Because, proteomic fingerprinting using the MALDI-TOF MS is an approach for fast, cost-effective species identification. All specimens were classified to species level indicated by low intraspecific and high interspecific unpredictability.

Sivakumar *et al.,* (2013) studied the Molecular phylogenetic analysis of freshwater cyclopoid copepods *Thermocylops decipiens* using 18S rDNA from Karappakkam Temple tank, Chennai. Study deals with the evolutionary relation among 12 species of freshwater cyclopoid copepods. Among 12 species of freshwater cyclopoid copepods, *Mesocyclops edax* and *Mesocyclops darwini* were single phyletic group in UPGMA method. By Maximum Likelihood analysis of *Mesocyclopos thermocyclopoides*, confident limit was 1.9870–7.30265 with positively significant at $p<0.05$ level and the distance compared with other species was 4.643516–5.873569. This study indicated the relationship of *T. decipiens* is close by related to other freshwater cyclopoid copepods *T. crassus* followed by *M. darwini* in both Phylognetic trees.

New species and its molecular and morphological affinities of genus Schizopera (Copepoda, Harpacticoida) in the Pilbara region of Western Australia

reported by Karanovic and McRae (2013). *S. cooperi* sp of this genus was a new documentation in this region. mtCOI partial sequences proposed that this new species has no close relatives among Western Australian congeners, molecular data for the apparently closely connected *S. weelumurra* are missing. This molecular data showed the relationship between *S. cooperi* and three congeners from the Yilgarn region. 28S rDNA and the ITS1 and ITS2 regions are useful markers for inferring the phylogenetic relationships between families of copepods and within the Cyclopidae family and related genera.

Phylogenetic analysis was studied by Zagoskin *et al.,* (2014) for 16 copepod species using ITS1/ITS2 rDNA from Russia. ITS1/ITS2 rDNA regions of cyclopoid copepods recommended that the *Mesocyclops, Thermocyclops*, and *Macrocyclops* genera share multifaceted evolutionary relationships. This study exposed that the ITS1 and ITS2 regions may have different phylogenetic signals.

Tomislav Karanovic *et al.,* (2014) analysed the copepod Stenhelia through morphological and molecular affinities using mtCOI gene. mtCOI gene sequences were obtained and this was the first genus in this region. Constructed phylogenies confirmed earlier postulated monophyly of Stenhelia and polyphyly of the closely related genus Delavalia Brady, 1869. This study showed the mtCOI is an effective tool to identify the closely related species.

DNA Barcoding of Marine copepods were studied by Cornils *et al.,* (2014). They analyzed 800 new barcode sequences for 63 copepod species. Sequences originated from 195 different taxa including 71 genera, 37 families and 4 orders. Of the 1,381 total sequences, 1,354 belonged to the Order Calanoida. This study revealed mitochondrial cytochrome c oxidase subunit I (COI) gene have new appreciation for

the strengths and weaknesses of this genetic marker for species assignment of planktonic copepods.

Saravana Bhavan *et al.,* (2016) investigated the diversity and molecular characterization of zooplankton through mt-COI gene from four perennial lakes of Coimbatore, India. Each species has obtained 650bp and the sequences showed 75-94% similarity. These four zooplankton species (*Asplancha intermedia*, *Moina micrura*, *Mesocyclops edax* and *Cypris protubera*) are genetically distinct but are closely related with mtCOI gene.

Seong-il Eyun (2017) resolved the phylogenetic relationship among and within four copepod order using 24 nuclear protein coding genes. Copepod has a close resemblance to the clade of Malacostraca and Thecostraca but not to Branchiopoda. In this study the phylogenomic analysis showed that three podoplean copepod orders are evidently clustered as a monophyletic clade.

5. Studies on Copepod culture

Various researchers have attempted to mass culture the different species of copepods. Zillioux (1969) was probably the first to describe a continuous culture system for planktonic copepod. Preetha and Altaff (1996) have studied the egg production rate of freshwater cyclopoid *Sinodiaptomus* (*Rhinediaptomus*) *indius* using different feed types and observed that a maximum of 90% females reared egg sacs when fed with algal feeds.

Muthupriya and Altaff (2004) have studied the fecundity rate of cyclopoid copepod *Mesocyclops thermocyclopoides* using mixed algae and baker's yeast.

Mehraj Ud Din and Altaff (2010) have cultured cyclopoid copepod *Thermocyclops decipiens* by using mixed microalgae and obtained a maximum population density of 5527 ind./L.

Mehraj Ud Din and Altaff (2010) Cultured *Ceriodaphnia cornuta*, using chicken manure as fertilizer. This study reveals that dehydrated chicken manure-fertilized medium at 700ppm appears to be a suitable medium for successful culture of *C. cornuta* to high density of 10,232 ind/L.

War and Altaff (2014) have studied Utilization of poultry excreta for high density production of *Daphnia carinata* (King 1853) and cultured for 21 days using poultry excreta to fertilise the medium at the rate of 500 ppt and maximum density of 5633.32 ± 88 Ind./L was recorded on 11th day of culture in the tanks, where, the feed was administered with 25% dosage followed by 50% dosage (1894.44 ± 9.68 Ind./L) and 75% dosage (1103.55 ± 17.80 Ind./L) of the initial dosage (500 ppt).

Altaff and Janakiraman (2015) have cultured cyclopoid copepod *Apocyclops dengizicus* using chicken manure fertilized medium and achieved a total population density of 6000 ind./l.

Rajkumar and Rahman (2016) have cultured the Calanoid Copepod, *Acartia erythraea* and Cyclopoid Copepod, *Oithona brevicornis* with various microalgal diets. A maximum population density obtained in mixed algae, recorded the maximum density of *A. erythraea* with 4583 nauplii, 2097 copepodites and 1429 adults per litre while the maximum density of *O. brevicornis* was 4211 nauplii, 1249 copepodites and 710 adults per litre.

Jasmine *et al.,* (2016) have observed the laboratory culture of the harpacticoid copepod *Euterpina acutifrons* (Dana, 1847) using different diet like *Isochrysis galbana, Tetraselmis gracilis, Chaetoceros calcitrans* and *Chlorella marina.* Mixed

diet of *I. galbana* and *C. calcitrans* showed maximum adult production followed by a diet of *T. gracilis*.

6. Studies on Biochemical composition of Zooplankton

Biochemical compositions of zooplankton were studied by different authors in different regions of India viz., Goswami *et al.,* (1981) from Andaman Sea analysed biochemical composition and recorded that the protein was the dominant constituent in mixed zooplankton and biochemical constituent values were low in forms with a high-water content.

Kumari and Achuthankutty (1989) reported the standing stock and biochemical composition of zooplankton in the North Eastern Arabian Sea. The variations in the biochemical composition between the coastal and oceanic zooplankton, the standing crop estimates clearly indicated that the coastal region is more productive than the oceanic region.

Sreepada *et al.,* (1992) Studied biochemical composition and caloric potential of zooplankton from Bay of Bengal. The variations in the biochemical constituents between the oceanic and neretic regions, the proteins form a major component and serves as an important metabolic reserve and protracted availability of phytoplankton in tropical water. Zooplankton doesn't have an extensive storage of carbohydrate and lipid.

Kumari and Goswami (1993) reported the biomass and biochemical composition of zooplankton from northwest Bay of Bengal during January 1990. Biomass, proximate composition, organic carbon and calorie content of assorted zooplankton from the surface waters were studied. Day and night stations revealed significant difference in biomass (displacement volume, dry wt and organic carbon)

whereas at coastal and oceanic stations irrespective of day and night the difference was significant only in dry wet values.

Stottrup *et al.,* (1999); and Sargent *et al.,* (1999a) reported that the nutritional qualities of copepods include high levels of protein and a rich source of essential fatty acids, especially Highly Unsaturated Fatty Acids (HUFAs) such as Docosahexaenoic Acid (DHA, 22:6 n-3) and Eicosapentaenoic Acid (EPA, 20:5 n-3).

Arts *et al.,* (1999) recorded lipid composition in freshwater zooplankton with selected ecological and physiological aspects and found that the lipids are key players in phenomenon of allelophathy and also act as chemical deterrents to foil zooplankton feeding. The ratio of storage to membrane lipids, maternal lipid investment, and the tracking of visible lipid energy stores are potentially useful indices of stress in zooplankton.

Biochemical compositions of zooplankton were studied by Goswami *et al.,* (2000) in west coast of India.

Nageshwara and Rathnakumari (2002) in Visakhapatnam harbour waters studied the biochemical composition and energy content of zooplankton and found that protein formed the major component followed by organic carbon, carbohydrate and lipid. Higher values of these constituents observed during high saline premonsoon and postmonsoon periods when the population densities of copepods, tintinnids, decapods, chaetognaths were high.

Safiullah Aman and Kareem Altaff (2004), have studied the biochemical profiles of a calanoid, *Sinodiaptomus (Rhinediaptomus) indicus,* and a cyclopoid, *Mesocyclops aspericornis*, from a natural pond for a period of one yr. In *S. (R.)*

indicus, moisture, protein, lipid, carbohydrate, ash, and amino acid contents were 81.1%, 68.1%, 8.9%, 19.11%, 3.2%, and 56.2%, respectively; while in *M. aspericornis*, the values of these parameters were 82.4%, 69.0%, 12.4%, 13.97%, 4.5%, and 62.9%, respectively. Fatty acid content was higher in *M. aspericornis* (102.38%) than in *S. (R.) indicus* (42.87%).

Perumal *et al.,* (2009) have studied biochemical composition of wild copepods, *Acartia spinicauda* and *Oithona similis*, from Parangipettai coastal waters in relation to environmental parameters. Percentage composition of protein, lipid, carbohydrate and amino acids of copepods, *Acartia spinicauda* and *Oithona similis* of the principal biochemical constituents were analysed. It was recorded as protein formed the major component followed by lipid and carbohydrate. Biochemical composition analysis of wild copepods indicated their nutritional rank. Totally 16 amino acids were observed in these wild copepods, with threonine, glutamic acid, alanine, aspartic acid, serine, valine and methionine as the dominant ones.

Jagadeesan *et al.,* (2010) reported the Biomass and Biochemical Composition of Zooplankton along the Arabian Sea, West Coast of India. Biomass (ml/100 m^3) varied from 25.2 to 42 ml/100 mg Protein formed the major component, varied from 21.07 to 40.73%. Lipid content ranged from 11.02 to 19.61 % and carbohydrate (5.83 to 14.98%).

Manickam *et al.,* (2017) recorded the evaluation of nutritional profiles of wild mixed zooplankton in Sulur and Ukkadam Lakes of Coimbatore, South India. It was revealed that the wild mixed zooplankton has more desirable dietary nutritional characteristics as larval diets than traditional live feed Artemia nauplii. Because the

mixed zooplankton having high total protein content followed by carbohydrate, lipid, moisture and ash.

7. Toxicity studies in Zooplankton

Baudouin and Scoppa (1974) Studied acute toxicity of various metals on freshwater zooplankton. The toxicity of metals with some of the physico-chemical characteristics showed significant correlations ($P < 0.05$) between median lethal concentration to Cyclops or Daphnia and the solubility product of metal sulfide.

Gebhardt (1976) evaluated the toxic effects of Cd, Cu, and Hg on *A. salina* growth and reproduction using a static renewal test with medium and food replacement every 3 days.

Jorgensen and Jensen (1977) observed that when *Artemia salina* was exposed to Cu ions, the hatching rate is much more susceptible than mortality, obtaining EC50 values at least 100 times below the analogous LC50.

Day (1988) reported the acute, chronic and sublethal effects of synthetic pyrethroids on freshwater zooplankton and find toxicity level in laboratory range from 0.12 to 5.0 µg/L for Cladocerans and copepods. Lower concentrations of pyrethroids (≤0.01 mg/L) reduced reproduction and rates of filtration of food by daphnids.

Snell and Persoone (1989) conducted the acute toxicity bioassays using rotifers *Brachionus rubens* in Belgium waters and showed that the 24hrs acute test at 25^{o} C, hatching resulted commencement of after 17h and by 25h, 40% of cysts were hatched. The average haching percentage for *B. rubens* cyst were 53%. Six compounds were assayed and had the following toxicity rankings: copper > NaPCP > cadmium > SDS > free NH3 > malathion. *B. rubens* was at least twice as sensitive as *Brachionus plicatilis* to all toxicants tested except malathion. The precision of the *B. rubens* acute toxicity test is about 3 times better than that of Daphnia.

Baird *et al.,* (1990) determined the LC50 values of eight clones of *D. magna* in acute toxicity tests and their LoEC (lowest effective concentration) in chronic toxicity tests for cadmium and 3,4-dichloroaniline (DCA), and found a large difference in sensitivity among the clones. Standardized acute and chronic toxicity tests have been intensively conducted using cladocerans, in particular the species Daphnia (OECD, 1981; ASTM, 1994).

Sanna Koivsito (1995) reported the ecological representative of zooplankton species *Daphnia magna* for toxicity analysis. But it is subjected to controversy that their finding of the the species *D. magna* is not a representative zooplankton species; hence, it is possible that the results of *D. magna* toxicity tests may give misleading information when they are extrapolated to natural zooplankton communities from the ecological point.

Folt *et al.,* (1999) tested the effects of sodium dodecyl sulfate under low food availability and high temperature on Daphnia and found that a combination of these stressors was more harmful than either one alone.

Hook *et al.,* (2000) studied sublethal effects of silver in zooplankton and anaylsed the importance of exposure pathways and implications for toxicity testing and compared the response of marine and freshwater crustacean zooplankton to silver through dissolved and food exposure. Ag is toxic at concentrations of 400 nm for marine copepods and 100 nm for freshwater cladocerans, concentrations far greater than those in most waters.

Brix *et.al*. (2003) determined the chronic (28d) toxicity of as (Na_3AsO_4) to *Artemia franciscana* measuring the effects on growth, reproduction, and survival under intermittent flow-through conditions considering a full life-cycle approach.

Caldwell *et.al*., (2003) compared hatching success and larval mortality of *Artemia salina* exposed to diatom extracts and aldehydes, showing that hatchability was less sensitive endpoint.

Rotini *et al.,* (2015) compared the results of hatching, acute mortality and chronic mortality tests on DEG and SDS and ranked the endpoints as follows: acute mortality < hatchability < chronic mortality.

Manfra *et al*., (2015) considered a 14-day test with *Artemia franciscana* to investigate the toxicity of DEG (Diethylene glycol) as an anticorrosive agent frequently used in offshore oil drilling activities.

Marus *et al.,* (2015) designed a New Toxicity Test using the freshwater Copepod *Cyclops vernalis*. Tests were performed with a range of concentrations of total dissolved solids (TDS) to develop a site-specific water quality objective. *C. vernalis* having less sensitive to TDS compared to *D. magna* and *C. dubia*, but similarly sensitive to an alga, a diatom, a rotifer, a chironomid, and two fish species. No adverse effects on survival or growth of *C. vernalis* at TDS concentrations up to 1500 mg/L was found Kennedy *et al.,* (2016) dredged material evaluations and did a review of Zooplankton toxicity test methods for marine water quality evaluations.

8. Studies on Immune mechanisms in Copepod

Even the simplest organisms' posses a defense system to prevent an infection or to limit the growth of the pathogen or parasite. About 50 species of microsportidian parasites have been described from copepods (Bronnvall and Larsson, 2001). Copepods are important intermediate hosts for nemetodes and cestodes.

Briggs (1976) found in experiments on copepod immunity to *Anemonia sulicata* and documented that certain copepods have an immunity to the toxin producing hematocystss found in the tentacles of the Cnidarians, while others are susceptible. However, the biology of the copepods in terms of protection against nematocycst toxins was not studied or understood (Humes, 1985).

Kurtz and Franz (2003) demonstrated remarkable degree of memory in Copepod immunity to tapeworms. Hence the presence of specific immunity was assumed and was correlated with lectins (Simpson, 2005).

Kurtz (2006) has reviewed the host parasite interactions between the cyclopoid copepods, *Macrocyclops allidus* and tapeworm parasite *Schistocephalus solidus*.

Though the Zooplankton immune system has not yet been explored, the crustacean immune mechanisms were well studied. Alpuche (2009) has reviewed the different immunity mechanisms in crustaceans. The different mechanisms registered include pattern recognition proteins, antimicrobial proteins, phagocytosis, encapsulation, lectins and clottable proteins. Batishella *et al.,* (2009) discussed the role of lectins in the Cellular immune response and the role of ProPo system in crustacean immunological mechanisms.

Edlund *et al.,* (2012) showed Cardinium bacteria as an endosymbiont in copepod as a first record and also induced antibiotic resistance strains caused reproductive manipulations in the copepods. However, the immune mechanisms behind the manipulation were not studied.

Hence in the present study an attempt was made to identify the presence of hemocytes and any immune mechanism of them based on the clue that Copepods are

micro crustaceans and thus should posses the same immune mechanisms found in macrocrustaceans.

9. Scientific studies on Cow urine

Copper has the power to destroy diseases and act as an antidote. Cytokines and amino acids might play a role in immune enhancement. Gomutra alone has got all such chemical properties, potentialities and constituents that are capable of removing all the ill effects and imbalances in the body (Chauhan and Singh, 2001). Immunnomodulatory agents are used to either suppress or stimulate the immune response. Cow's urine has immunostimulant activity in plants and animals, (Prabhakar *et al*., 2004).

Disinfectant prepared using cow urine and plant extracts (Neem, Tulsi, Ritha and Pine oil) is biodegradable and ecofriendly with good antibacterial action (Mandavgane *et al.,* 2005). Anti-leishmanial effect of cow urine while searching for an alternative for fetal calf serum (FCS), which is used as a growth supplement in the media for *Leishmania donovani*, a causing Leishmaniasis was described by Singh (2005).

Banga and Chauhan (2005) Suggested that Cow urine has immense potential of being used as an immunomodulator particularly along with antibiotics and/or vaccines in order to enhance their activity. However, its palatability in crude form as it is being prepared and marketed by several organizations, is not much accepted in the society. Therefore, the efforts are being made to prepare the dry form of cow urine without losing its activity but changing the delivery system and in this direction the scientists of JD Lab at CDDL, IVRI have got success and their preliminary research

has shown encouraging results. In future, it can be given as a 'biovaccine' to protect animals from various diseases.

Since the ancient period, the cattle are very good friends to human life. The indigenous cattle, mainly inhabit the Indian subcontinent. It is thought to be world's oldest domesticated cattle. Also, historically it is proved by the fact that humped cattle remain were found in Mohanjodaro site of Indus Valley indicating their presence in India even before the arrival of Aryans. Presently cow rearing is an important source of income and an enterprise, which enables poor and landless farmers to earn income using common property resources and land (Chauhan, 2007).

Golder *et al.,* (2007) conducted a study with a view of converting urine into bio-wealth in the form of zooplankton. The nutrient potentials of human urine, human–cow mixed urine, cow urine, vermincompost, poultry droppings, mixed wastes (vermin–cow–poultry) and cow dung were evaluated for the mass culture of zooplankton *Moina micrura*. Total number of *Moina micrura* enumerated in the culture tank, related with offspring production per life span, was maximum in case of human urine treatment, followed by human–cow mixed urine, cow urine, vermin-compost, poultry droppings, vermin–cow–poultry wastes and cow dung. The relationship between the total offspring production per female per life span and the nitrogen content of water in different treatments implied that human urine was an excellent liquid waste followed by cow urine that can be used for the mass production of zooplankton *Moina micrura* required for larval and post larval rearing of commercial fishes.

Natural products of plant and animal origin offer a vast resource of newer medicinal agents with potential in clinical use (Ismail *et al.,* 2009). Recent researches showed that cow urine enhances the immune status of an individual through activating the macrophages and augmenting their engulfment power as well as bactericidal activity. Chauhan *et al.,* 2009 stated in his results anticancer potential of cow urine therapy has been reflected by several case reports and practical feedback of patients for the treatment of cancer. Cow urine enhances the immunocompetence and improves general health of an individual; prevent the free radical's formation and act as anti-aging factor; reduces apoptosis in lymphocytes and helps them to survive; and efficiently repairs the damaged DNA, thus is effective for the cancer therapy. Experimentally it has been proved that among all sorts of urines, the urine of the Indian cows is most effective.

Rajesh (2016) states that in context to urological disorders, cow urine is extremely beneficial because it provides some of the most basic vitamins and minerals into the body and also Cow urine therapy is helpful in cancer, diabetes, AIDS, asthma, psoriasis, eczema, blood pressure, heart disease, prostrate, piles, asthma, esnophilia, cough, phlegm, varicose veins, dismonerrhoea, cholesterol, chest pain, aids, migraine, headache, tension, constipation, thyroid, eczema, ringworm, itching and other skin problems, liver disorder, kidney problem, achne.

The Times of India, and other media outlets on 2016 June 28, reported that researchers at Junagadh Agricultural University (JAU) in Gujarat, had found gold particles in the urine of some cows from the Gir region of the state. This elemental analysis, undertaken with the objective of finding metabolites and toxins, was reported to have found 3-10 mg of gold per litre of cow urine, as well as 5,100 known

compounds. Some 338 of these compounds have been claimed to be of ‘immense’ medicinal value, and in line with the ayurvedic descriptions.

Gosavi *et al.,* (2011) Singla and Kaur (2016) studied in redistillate cow urine the concentration of volatile fatty acids is about 1500mg/dl these fatty acids and other antioxidants might cause the observed protective effects.

DESCRIPTION OF STUDY AREA

Kumbakonam is a town and a special grade municipality in the Thanjavur district in the southeast Indian state of Tamil Nadu. The town is bounded by two rivers, the Kaveri River to the north and Arasalar River to the south. Kumbakonam is located at 10.97°N 79.42°E. The town comprises above 40 ponds.

Sampling locations were selected based on the preliminary survey carried out in the nearby environs Kumbakonam. There are many freshwater bodies in and around Kumbakonam. They have been impacted by the anthropogenic activities such as encroachment, dumping of solid waste and pollution by letting untreated domestic sewage. Review of literature on the zooplankton diversity of freshwater bodies in and around Kumbakonam reveals that there is no published report on this subject in recent time. Hence it is felt that studies on culture of copepod on freshwater bodies have to be pursued. Accordingly, these three ponds were selected for the collection of zooplankton. The sampling survey covered geographically distinct regions in the urban zones of Kumbakonam. Following are the selected ponds.

1. Mathi Pond (Urban limits)
2. Pidari Pond (Urban limits)
3. Sei Pond (Urban limits)

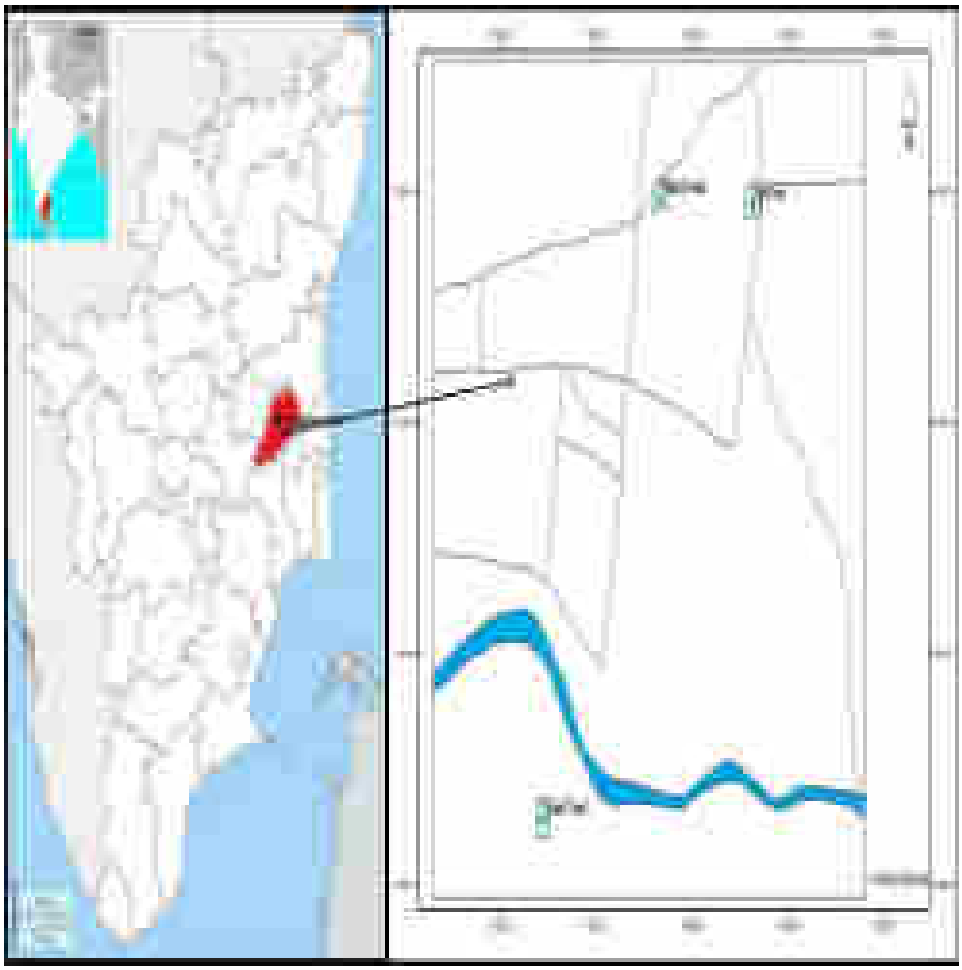


Fig 1. Map showing the sampling areas at kumbakonam

1. **Mathi Pond**

Mathi pond is located in Kumbakonam (10° 56' 45.82" North, 79° 22' 48.81" East), Thanjavur district of Tamil Nadu. The pond, selected for this work, is a perennial freshwater, sewage-fed pond. It is more than fifty years old pond, almost squarish in shape with irregular shore line. The sources of its replenishment are rain water, waste water and sewage from surrounding localities which are fed through inlet drains present on the corners of the pond.

Considerable litter is also deposited in the pond bottom. Pond is partially infested by the surface macrophyte, water hyacinth (*Eichhornia crassipens*). The pond

is used as drainage basin into which drainage water sweeps from the surrounding localities, and also for bathing purpose of the cattles. Pond receives large quantities of human excreta, animal dung and sewage from nearby settlements, contributing organic nutrients to the pond. Bottom of the pond is more or less flat and contains mostly mud, stones, parts of dead plankton and deposited, decomposed and partially decomposed litter. The colour of the bottom material ranges from grey to brown and blackish during different seasons of the year. The water of the pond is turbid and dull green in colour. The pond is being used as an intensive fish culture site for *Oreochromis mossambicus*, *Labeo rohita*, and *Channa striata.*

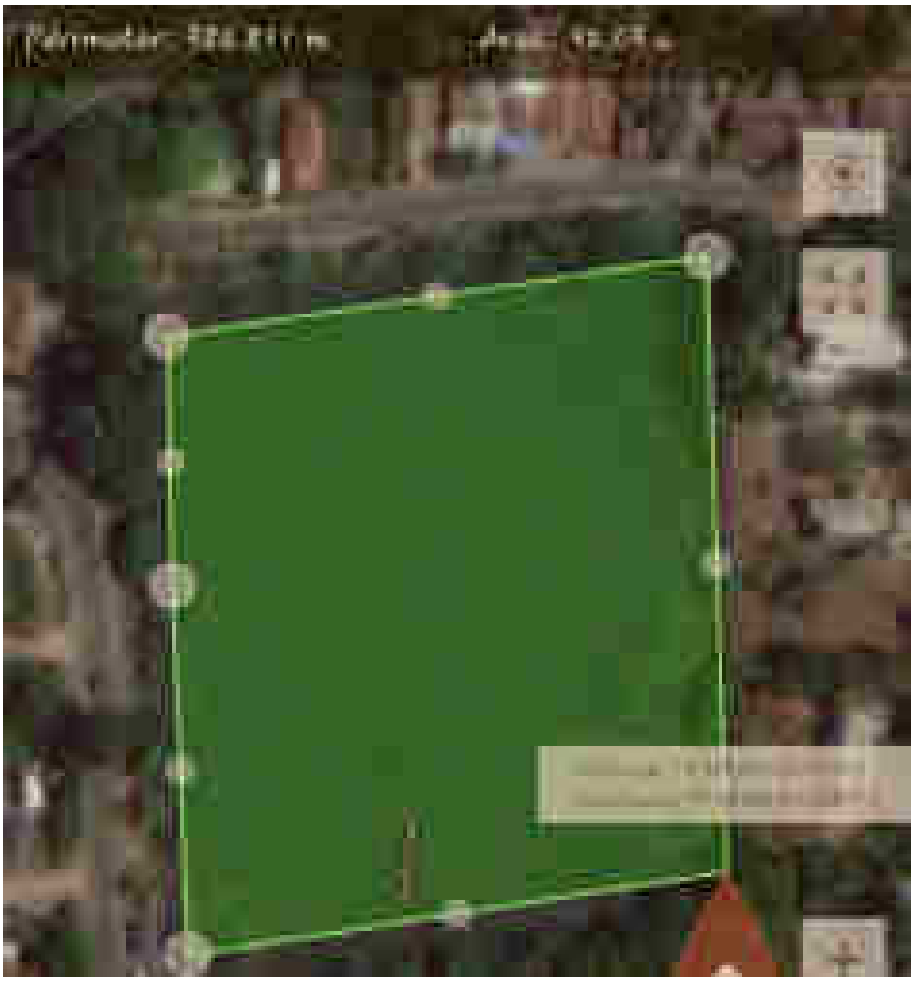


Fig 2. Satellite View of Mathi Pond

Plate 5. Station 1 - Mathi Pond

Plate 6. Collection of Water and Zooplankton Samples from Mathi Pond

2. Pidari Pond

Pidari pond (10° 57' 39.63" North, 79° 23' 13.36" East) is located near fish market at Kumbakonam, Thanjavur district of Tamil Nadu. The pond is rectangular with concrete stairs along the circumference and is surrounded by fish culture and National road. Though the pond is believed to be sacred, yet it is not free from anthropogenic influences. This water body is also affected by encroachment and discharge of untreated domestic sewage. This pond is being used as a dumping site for fish waste from the local fish market. Apart from this it is also being used for culture of *Oreochromis mossambicus, Labeo rohita*, and *Channa striata.*

Fig 3. Satellite View of Pidari Pond

Plate 7. Station 2 - Pidari Pond

Plate 8. Collection of Water and Zooplankton Samples from Pidari Pond

3. Sei Pond

Sei pond is located in Kumbakonam (10° 57' 39.18" North, 79° 23' 32.40" East), Thanjavur district of Tamil Nadu. The water body is man made and the pond has suffered due to exploitation by residents from the locality and dumping of waste in addition to letting untreated sewage water into it. *Oreochromis mossambicus*, *Labeo rohita*, *Cirrhinus mrigala, Catla catla* and *Channa striata* are cultured in this pond by local fish farmers.

Fig 4. Satellite View of Sei Pond

Plate 9. Station 3 - Sei Pond

Plate 10. Collection of Water and Zooplankton Samples from Sei Pond

I. Analysis of physico-chemical parameters

Samplings were conducted every month to record the physico-chemical, and zooplankton characteristics. Rainfall data was obtained from the Meteorological unit located at Thanjavur District, Tamil Nadu, India. The water samples were taken in sterile, wide-mouth, screw capped glass bottle vertically between 1 to 4 meter depth with few meter of distance between samples from surface and bottom using Van Dorn Sampler during early morning hours (6 -7 AM) and transported to the laboratory under ice cold condition, and subjected to analyses on the same day of collection. Air and water temperature were measured on the spot. Salinity, pH, electrical conductivity (EC), total dissolved solids (TDS) and dissolved oxygen (DO) were estimated by using "Microprocessor based Water & Soil Analysis Kit Model - 1160" (Systronics).

Biological Oxygen Demand (BOD) was found after three days of incubation at 27^{o}C, while other parameter such as Chemical Oxygen Demand was determined by titration method (APHA, 2005). The water samples were filtered using a Millipore filtering system and analyzed for dissolved inorganic nitrite, nitrate and reactive silicate by adopting the standard procedures described by (Strickland and Parsons, 1972) and are expressed in µM. The recorded data in every month were grouped based on four seasons, viz., summer, pre-monsoon, monsoon and post-monsoon (Manickam *et al.,* 2014).

II. Plankton Studies

i) Collection of Plankton Samples

The plankton samples were collected during early morning between 6.00 and 7.00 AM, in the second week of every month for one year from January 2017 to December 2017.

a) For Qualitative Analysis (Species composition)

The plankton samples were collected using Towing- Hensons's standard plankton net (150 µm) by towing horizontally at surface for about 10 minutes with a uniform speed (Davis, 1955).

b) For Quantitative Analysis (Population density)

For the quantitative analysis of phytoplankton, 100 liters of water were filtered through plankton net made up of bolting silk cloth (150 µm) using a 10 liters capacity plastic container. Immediately after filtering out the water, the plankton biomass was transferred to plastic container with 5% of neutralized formalin and subjected to microscopic analysis (Davis, 1955).

ii) Preservation

The collected zooplankton samples were filtered with the help of filter to remove the water and then the samples were transferred to polyethylene bottles (100ml) filled with 5% of formalin (10ml), the aqueous solution of formaldehyde (Davis, 1955).

iii) Separation and Temporary mount

Zooplankton was segregated group wise, which includes Rotifera, Cladocera, Copepoda and Ostracoda. They were identified and separated under a binocular stereo zoom dissection microscope using a fine needle and brush. Individual species of

plankton was mounted on microscopic slides on a drop of 20% glycerin after staining with eosin and rose Bengal stains (Manickam, 2014).

iv) Identification of Zooplankton

The identification of zooplankton was made by referring the standard manuals (Sharma and Michael, 1987; Murugan *et al.,* 1998; Altaff, 2004) and text books (Edmondson, 1959; Battish, 1992; Reddy, 1994) using a compound microscope and photomicrographs were taken using inverted Biological Microscope attached with a camera (Nandakumar *et al.,* 2015).

v) Quantitative analysis

One ml of sample was taken with a wide mouthed pipette and poured into the counting cell of the Sedgwick Rafter Counting slide by following Santhanam *et al.,* (1989) and counted under light microscope.

vi) Statistical analysis and diversity indices

Statistical analysis was done in MS Excel software. Correlation coefficient (r) was calculated for zooplankton and physico-chemical parameters and analysis of variance (F) tests were made for hydrological parameters in relation to stations and seasons. Shannon and Weaner's species diversity index (H'), Species richness (d), and Evenness index (J') were calculated using the software PRIMER (Version 6).

III. Culture of Copepods

i) Isolation of Copepods

The collected water samples were filtered through super imposed sieves to separate copepods from other zooplankton and also to remove nauplii and other larval

forms. From the sieved samples, copepods were observed under a dissecting binocular microscope and healthy adults of individuals were isolated and copepod species was morphologically identified under the microscope. The isolated copepods were maintained in a 500 ml beaker containing tap water.

ii) Developing pure culture of Cyclopoid copepods

To begin with, from the copepods isolated, *Cyclopoid sp.* was identified under microscope (10x) using a broad mouthed pipette and brush practical manual (Lynne M. Witty, 2004). The Cyclopoid species were isolated and inoculated into another 2-liter container and fed with yeast media. The yeast was dissolved in clean and filtered freshwater to avoid dust particles and other waste materials. Feeding was done once in three days or whenever the water in the culture tank becomes clear. Excess feed would cause water quality problems which would lead to the mortality of copepods; hence care was taken to avoid excess feeding (Nandakumar *et al.,* 2015).

iii) Yeast Preparation

The dry yeast used as feed in the study was commercially obtained from market. The yeast media was prepared by diluting 0.1 g of yeast in 100ml of freshwater (Munirasu *et al.,* 2016).

iv) Copepod stock culture maintenance

From the diluted samples of isolated cyclopoid copepod adult male and female were isolated and initially stocked at 250 ml glass beakers for culture. Then the copepod mass culture was done in 20 l glass tank filled with tap water. The copepods were fed with yeast once in two days. The fecal pellets and debris were siphoned out

daily and the expelled culture water was replaced with filtered freshwater (Nandakumar *et al.,* 2015).

IV. Molecular level species identification

i) Sample preparation

For molecular identification, healthy cyclopoid copepod was handpicked and preserved in 95% ethanol. Before preserving the copepods, they were rinsed thoroughly in distilled water to eliminate dust particles and other impurities (Kasturirangan, 1963).

ii) Molecular identification

The molecular level species identification of the copepod predominantly occurred in the present study pond was done at RGCB (Rajiv Gandhi Centre for Biotechnology) Trivandrum Laboratory by the sequencing of 18S rRNA region. The detailed procedure adopted by the laboratory was given below.

Genomic DNA isolation from copepod

Genomic DNA was isolated from copepod sampleds using NucleoSpin® Tissue Kit (Macherey-Nagel) following manufacturer's instructions. A part of culture was taken in a microcentrifuge tube. A 180 µl of T1 buffer and 25 µl of proteinase K was added and incubated at 56^0C in a water bath until it was completely lysed. After lysis, 5 µl of RNase A (100 mg/ml) was added and incubated at room temperature for 5 minutes. 200 µl of B3 buffer was added and incubated at 70oC for 10 minutes. 210 µl of 100% ethanol was added and mixed thoroughly by vortexing. The mixture was pipetted into NucleoSpin® Tissue column placed in a 2 ml collection tube and

centrifuged at 11000 x g for 1 minute. The NucleoSpin® Tissue column was transferred to a new 2 ml tube and washed with 500 µl of BW buffer. Wash step was repeated using 600 µl of B5 buffer. After washing, the NucleoSpin® Tissue column was placed in a clean 1.5 ml tube and DNA was eluted out using 50 µl of BE buffer.

Agarose Gel Electrophoresis for DNA Quality and Quantity check

The quality of the DNA isolated was checked using agarose gel electrophoresis. 1µl of 6X gel-loading buffer (0.25% bromophenol blue, 30% sucrose in TE buffer pH-8.0) was added to 5µl of DNA. The samples were loaded to 0.8% agarose gel prepared in 0.5X TBE (Tris-Borate-EDTA) buffer containing 0.5 µg/ml ethidium bromide. Electrophoresis was performed with 0.5X TBE as electrophoresis buffer at 75 V until bromophenol dye front has migrated to the bottom of the gel. The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad).

PCR Analysis

PCR amplification reactions were carried out in a 20 µl reaction volume which contained 1X PCR buffer (100mM Tris HCl , pH-8.3; 500mM KCl), 0.2mM each dNTPs (dATP, dGTP, dCTP and dTTP), 2.5mM MgCl2, 1 unit of AmpliTaq Gold DNA polymerase enzyme, 0.1 mg/ml BSA, 4% DMSO, 5pM of forward and reverse primers and template DNA.

Primer used

Target	Primer Name	Direction	Sequence (5➔3´)
18S	1F	Forward	TACCTGGTTGATCCTGCCAGTAG
	4R	Reverse	GAATTACCGCGGCTGCTGG

PCR amplification profile

16S rRNA

95^0C - 5.00 min

95^0C - 30 sec

54^0C - 40 sec 40 cycles

72^0C - 60 sec

72^0C - 7.00 min

4^0C - ∞

Agarose Gel electrophoresis of PCR products

The PCR products were checked in 1.2% agarose gels prepared in 0.5X TBE buffer containing 0.5 µg/ml ethidium bromide. 1 µl of 6X loading dye was mixed with 5 µl of PCR products and was loaded and electrophoresis was performed at 75V power supply with 0.5X TBE as electrophoresis buffer for about 1-2 hours, until the bromophenol blue front had migrated to almost the bottom of the gel. The molecular standard used was a 2-log DNA ladder (NEB). The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad).

ExoSAP-IT Treatment

ExoSAP-IT (USB) consists of two hydrolytic enzymes, Exonuclease I and Shrimp Alkaline Phosphatase (SAP), in a specially formulated buffer for the removal of unwanted primers and dNTPs from a PCR product mixture with no interference in downstream applications.

Five micro litres of PCR product was mixed with 2 µl of ExoSAP-IT and incubated at 37^{o} C for 15 minutes followed by enzyme inactivation at 80^{o} C for 15 minutes.

Sequencing using BigDye Terminator v3.1

Sequencing reaction was done in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, USA) following manufactures protocol.

The PCR mix consisted of the following components:

PCR Product (ExoSAP treated) - 10-20 ng

Primer - 3.2 pM (either Forward or Reverse)

Sequencing Mix - 0.28 µl

Reaction buffer - 1.86 µl

Sterile distilled water - make up to 10µl

The sequencing PCR temperature profile consisted of a 1st cycle at 96^{0}C for 2 minutes followed by 30 cycles at 96^{0}C for 30 sec, 50^{0}C for 40 sec and 60^{0}C for 4 minutes.

Post Sequencing PCR Clean up

1. Master mix I of 10µl milli Q and 2 µl 125mM EDTA per reaction and master mix II of 2 µl of 3M sodium acetate pH 4.6 and 50 µl of ethanol were prepared.

2. 12µl of master mix I was added to each reaction containing 10µl of reaction contents and was properly mixed.

3. 52 µl of master mix II was added to each reaction.

4. Contents were mixed by inverting and incubated at room temperature for 30 minutes

5. Spun at 14,000 rpm for 30 minutes

6. Decanted the supernatant and added 100 µl of 70% ethanol

7. Spun at 14,000 rpm for 20 minutes.

8. Decanted the supernatant and repeated 70% ethanol wash

9. Decanted the supernatant and air dried the pellet.

The cleaned-up air dried product was sequenced in ABI 3730 DNA Analyzer (Applied Biosystems).

Sequence Analysis

The sequence quality was checked using Sequence Scanner Software v1 (Applied Biosystems). Sequence alignment and required editing of the obtained sequences were carried out using Geneious Pro v5.6 (Drummond *et al.,* 2012)

iii) Phylogenetic analysis

The sequences were initially edited in the Gene tool and Bio-edit software packages. Then, the edited sequences were submitted to NCBI database. DNA homology searches were performed using BLASTN 2.2.24 programs at NCBI.

V. Experimental studies on the Cyclopoid copepod culture

i) Survivablity in culture

The copepods cultured were subjected to survival experiments following Nandakumar *et al.,* (2015) to check the Suitability of the animals for further experiments. 10 healthy adults were handpicked and stocked in a glass beaker filled with 500 ml tapwater filtered with 1µ filter bag. They were starved for 24hours befor start of the experiment. The copepods were examined daily and counted for survival. The experiments were done in triplicate and carried out for a total of 14 days. The debris and fecal materials were removed daily by siphoning. The culture medium was replaced once in every two days to avoid the supremacy of poor water quality over survival of the copepods. The number of live copepods remained on the final day were counted for survival (Nandakumar *et al.,* 2015).

Plate 11. Experimental setup for survival of Copepod Species

ii) Cow urine collection

Six disease free cows as certified by the Veterinarian were ascertained for urine collection (Tag. No: 0206, 0177, 0184, 0468, 0133, 0201) from Goshala, Sri Vittal Rukmini Samsthan, Govindapuram near Kumbakonam. These animals were maintained under same conditions in a well-ventilated shed with the provision of

individual feeding and watering. Clean drinking water and feed was provided *ad libitum.* Animals were daily offered about 2 kg of pellet feed and with available green fodder and straw. The early morning first urine (4.00 to 5.00 am) was collected from the six cows, and was pooled together (Sattanathan and Venkatalakshmi, 2015) and transported to laboratory in airtight sterile containers.

Plate 12. Sri Vittal Rukmini Samsthan, Govindhapuram

iii) Cow Urine Distillate preparation

Following Arunkumar Sathasivam *et al.,* (2010) the collected cow urine samples were distilled at 50 - 60° C using distillation apparatus for 5 – 6 hours. The distillate collected was stored in sterile glass containers and was labeled as CUD with date. It was used for treatment on the same day without storage.

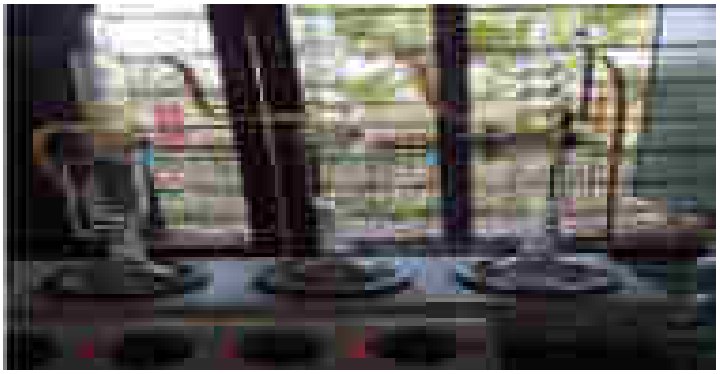


Plate 13. Cow Urine Distillation

iv) Toxicity of CUD on the Cyclopoid copepod

From the stock culture, only actively swimming adult animals were selected for the toxicity tests. Five concentrations of CUD Viz., 0.2%, 0.4%, 0.6%, 0.8% and 1% were used. Twenty animals were used in each of five concentrations and control. For each concentration animals were divided into two groups of 10 individuals (in duplicate). Well aerated tap water was used in all tests. Each test lasted 24hr and mortality was noted every 6, 12 and 24hr. Death of individuals was determined from absence of movement even when prodded gently.

v) Experimental protocol to study the effect of CUD on copepods

Five tanks with twenty liters of clean water was inoculated with a copepod population density of 500 Nos each. The zooplankton was fed with 2.5ml of yeast stock solution for every three days. The CUD was added in different concentrations Viz. 0.025%, 0.05%, 0.1% and 0.2% (fixed based on the LC_{50} value) in four experimental tanks. One tank was kept as control by adding equal volume tap water without CUD. The media is kept undisturbed for a period of seven days. After seven days water was changed. The plankton were well aerated with all culture requirements for 60days. After 60days, the survival, population density and biochemical composition of the CUD treated and untreated plankton groups were carried out. In the same experimental set up, 10 gravid female adults with viable egg sacs were kept inside a test tube of 20ml capacity covered with a nylon net was to hung with a wooden stick support to determine the nauplii production rate.

a) Survival

Survival at the end of experiment was calculated using the following formula.

$$\text{Relative Percent Survival} = n\text{-}1 \frac{\text{Number of Surviving copepods after CUD treatment}}{\text{Number of copepods dead due to CUD treatment}} \times 100$$

b) Population density

After Sixty days of post CUD treatment all the copepods including nauplii, copepodites and adults were filtered through 48µm sieve. The total copepods produced over the period of experiment were counted using sedgewick rafter counter under the 10X of microscope. The number of nauplii, copepodites and adult copepods in one ml sample were counted separately. The counting was done in replicates of five and the results were converted to per liter.

c) Nauplii Production Rate (NPR)

The gravid female copepods kept inside the test tube were examined at regular time intervals of two hours for the release of nauplii. Once the nauplii were released, the adult female was carefully removed from the test tube and the nauplii were counted under the microscope.

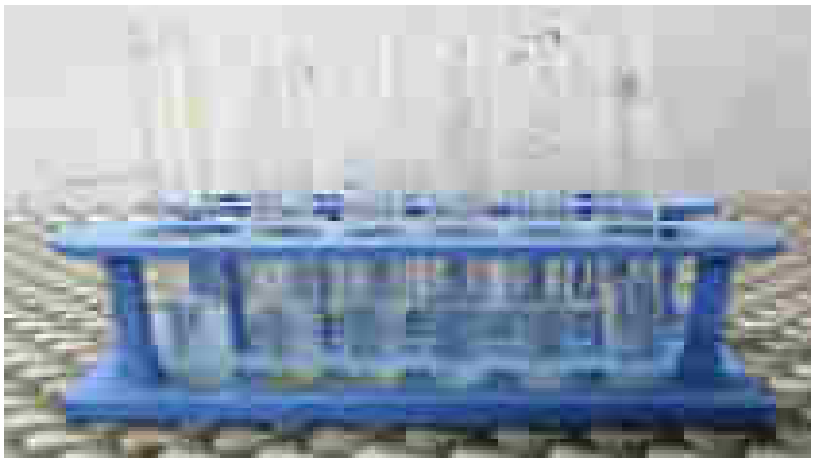


Plate 14. Experimental Setup for Nauplii Production Rate

d) Biochemical analysis

i) Protein estimation (Lowry's method)

Total protein was estimated by the method of Lowry *et al.,* (1951), using ethanolic precipitated sample. The blue colour was result of biuret reaction of protein with copper ions in alkali solution and reduction of the phophomolybdic-phosphotungstic of folin reagent by the tyrosine and trphtophan present in the treated protein, which was measured at 630 nm against a blank. Bovine Serum Albumin (BSA) was used as a standard. The result was expressed in percentage.

ii) Carbohydrate estimation (Anthrone method)

The concentration of total carbohydrate was estimated by the method of Roe (1955) using TCA – extracted sample. Carbohydrates are hydrolyzed into simple sugars by diluted hydrochloric acid (HCL) in hot acidic medium; glucose is dehydrated into hydroxyl-methyl furfural this compound reacts with anthrone and produced green coloured product, which was measured at 630 nm against a blank. Glucose was used as standard.

iii) Lipid estimation (Phospho – vanillin method)

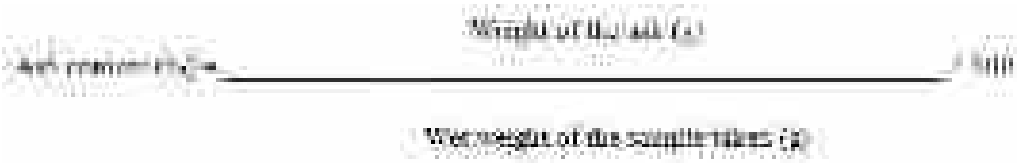
The total lipid was extracted with chloroform-methanol mixture following the method of Folch *et al.,* (1957) and estimated by the method for Banes and Blackstock (1973). Lipid reacts with vanillin in a medium of sulphuric acid (H_2So_4) and phosphoric acid (H_3PO_4) to form a pink-coloured chromogen, which is proportional to the lipid content of the sample, which was measured at 540 nm against a blank. Olive oil was used as standard.

iv) Moisture estimation

A known quantity of sample was taken and the excess moisture was removed using a filter paper (Rajendran, 1973). Then the sample was dried in a hot air oven at a constant temperature of 60^{o}C until the wet sample was dried completely. The moisture content was estimated by subtracting the dry weight of the sample from the wet weight of the sample. The percentage of moisture content was calculated as follows;

v) Ash estimation

The ash content was determined by burning oven – dried sample in muffle furnace at 550^{o}C as followed by the method of AOAC (1995). The result was expressed in percentage.



VI. Analysis of Copepod Immune System

i) Isolation of Hemolymph

20 mature adults were separated under a hand lens. Using a tissue homogenizer the planktons were crushed with Sterile physiological saline solution. The homogenate was then centrifuged in 600 rpm for 10 min. The tissue debris was

discarded. The supernatant was supposed to be the hemolymph (Praveena and Venkatalakshmi, 2019).

ii) Isolation of plasma and hemocytes

The hemolymph was centrifuged at 3000 rpm for 10 min which resulted in the separation of plasma and packed cells out of the hemolymph. The plasma is separated in an Eppendorf tube using a micropipette. The packed hemocytes were re-suspended in Sterile phosphate buffer saline for further analysis. This is a crude method of isolation of hemolymph, plasma and hemocytes attempted for the first time in zooplankton as there were no studies done so far in this (Praveena and Venkatalakshmi, 2019).

iii) Microscopic Examination of Hemocytes

Freshly packed hemocytes were stained with eosin and examined under light microscopy to determine cell size, cell shape and presence of granules under a magnification of 100X. A calibrated micrometer was used for cell measurements (Praveena and Venkatalakshmi, 2019).

iv) Isolation of Hemocyte lysate

The packed hemocytes obtained after centrifugation were resuspended in Sterile phosphate buffer saline 1 ml of sample was taken in an eppendroff tube and the cells were lysed and cell lysate by means of sonication at 20kHz for 10 minutes.

v) Estimation of total proteins in hemolymph and hemocyte lysate

The quantitative estimation of protein was determined by the method of Lowry *et al.,* (1951) using bovine serum albumin as the standard.

vi) Isolation and Determination of Molecular weight of Protein in hemolymph and hemocyte lysate by Electrophoresis

Polyacrylamide gel electrophoresis was done to separate protein. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was done to determine the molecular weight of the proteins present in the hemolymph and hemocyte lysate following the procedures adopted by Laemmli, 1970. The gel was stained with coomassie brilliant blue R250. The distaining was done using methanol and acetic acid in the ratio 5:1 (Bogovic, 2000).

The gel electrophoresis apparatus was washed thoroughly with 10% SDS to remove the impurities. Stacking gel and separating gel was prepared using the above explained procedure. Spacer was washed thoroughly and it was then placed in the gel plate. Gel was prepared by pouring the separating gel. Comb was placed immediately after pouring the stacking gel into the gel plate. The comb was removed carefully after the polymerization of stacking gel. The gel was kept in the gel tank and running buffer was added into the well. Then 20 µl of sample was isoslated into the well. Electrophoresis was carried out at 80v until the tracking dye reaches the end. The gel was separated from the gel plate carefully and it was stained in the staining solution for 3hrs. After 3hrs the gel was placed in the distaining solution so the excess stain gets removed from the gel so that the bands can be visualized clearly. The protein bands separated by the polyacrylamide gel electrophoresis were cut separately and isolated in physiological buffer solution (Laemmli, 1970).

vii) Antimicrobial activity of Proteins in hemolymph and hemocyte lysate

Antimicrobial activity of the plasma and hymolysate proteins with high molecular weight were determined using well diffusion method. It was performed by

sterilizing Muller Hinton agar media. After solidification, wells were cut on the Muller Hinton agar using a well borer. The test bacterial pathogens were swabbed on to the surface of Muller Hinton agar plates. Wells were impregnated with 25µl of the test sample. The plates were incubated for 30 min to allow the samples to diffuse into the medium. The plates were incubated at 30°C for 24 hr, and then the diameters of the zone of inhibition were measured in millimetre. Each antibacterial assay was performed in triplicate and mean values were reported (Lila ruangpan and Tendencia, 2004).

Water parameters

The physico-chemical parameters are suspicious as the most significant ideologies in the identity of the environment, eminence and kind of the water bodies (fresh, brackish, saline) for any aquatic biota. The results for the Physico-chemical parameters of the present study sites were presented as graphs using sigma plot 14 version.

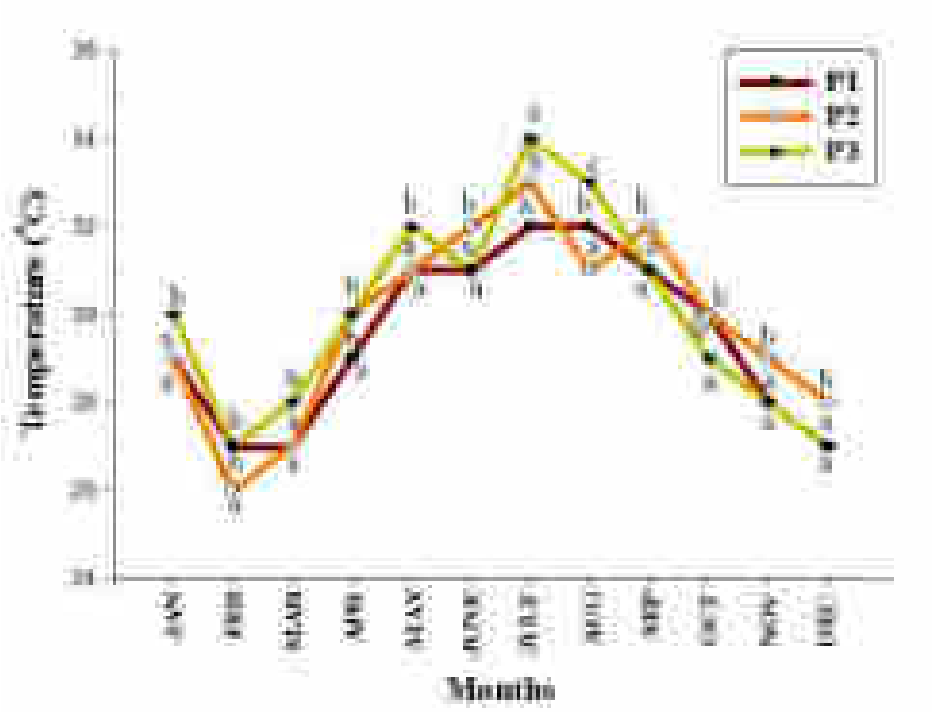
Rainfall

Fig 5. Meteorological Rain Fall data of Kumbakonam during January – December 2017

The study ponds Viz., Mathi pond (P1), Pidari pond (P2) and Sei pond (P3) are located in the same geographical area (i.e) Kumbakonam, Thanjavur District (Fig. 00; Chapter III). The rainfall during the study period in the study area showed a diverse seasonal trend of fluctuation. It was recorded maximum as 429.4mm in

November, 2017 and the minimum was 1.3mm in February, 2017 (Fig. 5). The average rainfall data over the year was found as 118.17mm.

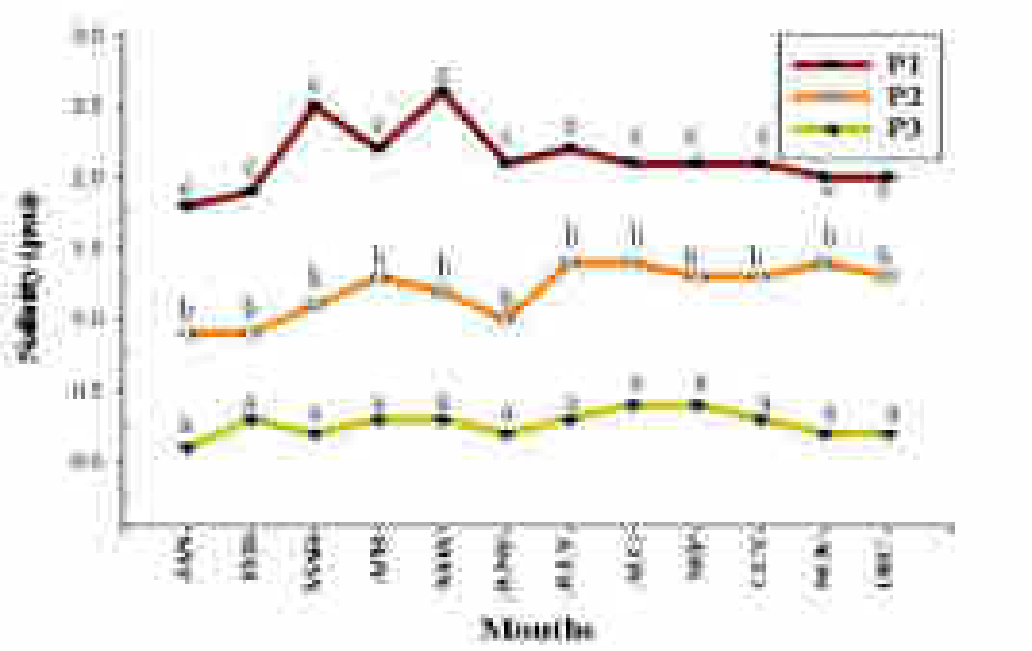
Fig 6. Water temperature of study ponds at Kumbakonam during January – December 2016; Each Point represents mean ± SE of 3 samples; a posteriori Holm-Sidak comparison of study ponds are shown with different alphabets representing significant difference (P<0.05)



Water temperature is an important asset that decides water fitness for human exploitation, industrial appliances and aquatic functioning. In the present study area the temperature varied from 26°C to 34°C. The minimum (27°C) was observed in station P1 during the month of February, March and December, 2017 and the Maximum (32°C) was observed during the month of July and August 2017. In Station P2 the minimum water temperature, 26°C was recorded in the month of February, 2017 and the maximum of 33°C in July, 2017. In station P3 the minimum of 27°C was

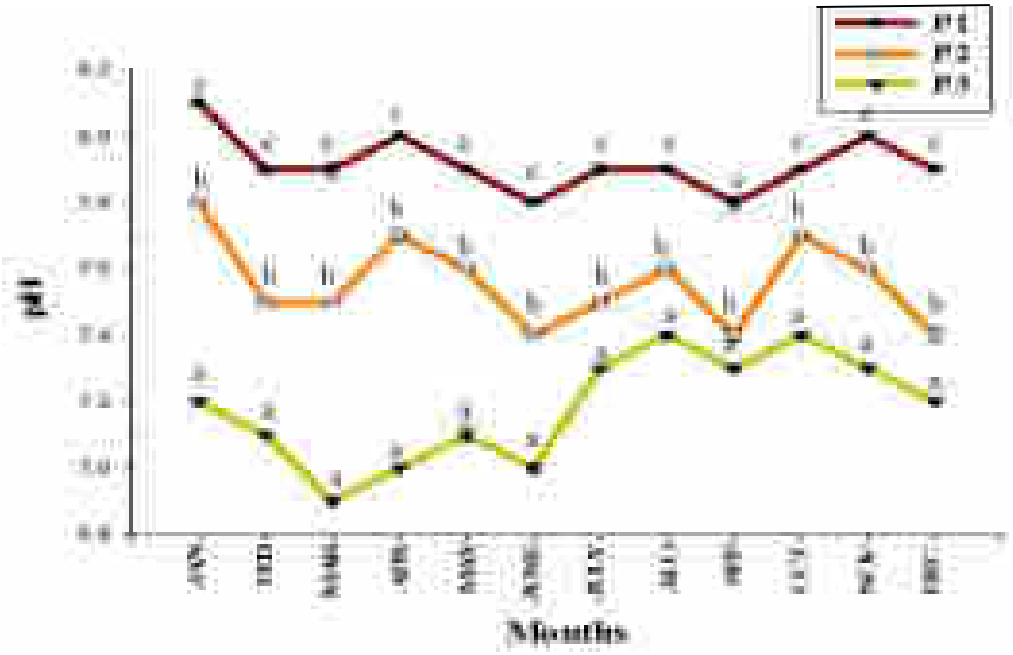
recorded in the month of February and December, 2017 and the maximum of 34°C was recorded in July, 2017. (Fig.6).

Fig 7. Salinity range of study ponds at Kumbakonam during January – December 2016; Each Point represents mean ± SE of 3 samples; a posteriori Holm-Sidak comparison of study ponds are shown with different alphabets representing significant difference (P<0.05)



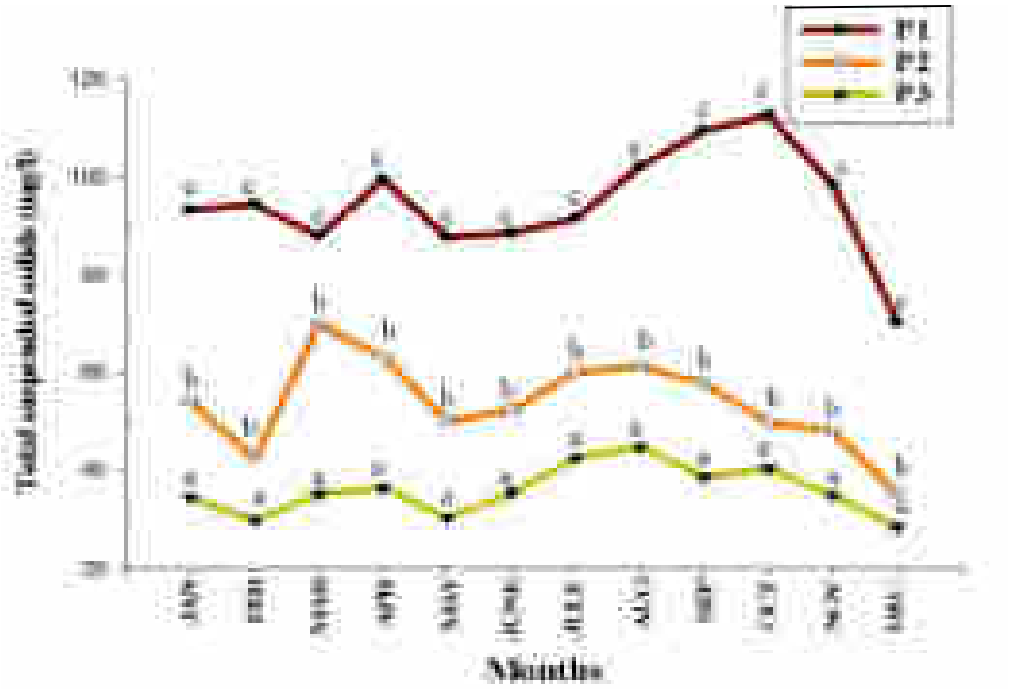
The minimum concentration of salinity 1.8psu was recorded in January, 2017 and maximum concentration of salinity 2.6psu was recorded in the month of May, 2017 at P1 station. In P2 station maximum 1.4Psu salinity was observed in the month of July, August and November. The minimum 0.9Psu salinity was observed in January and February, 2017. The minimum salinity of 0.1Psu was recorded in January and the maximum 0.4Psu salinity was recorded during the month of August and September, 2017 in P3 Station (Fig. 7).

Fig 8. pH limits of study ponds at Kumbakonam during January – December 2016; Each Point represents mean ± SE of 3 samples; a posteriori Holm-Sidak comparison of study ponds are shown with different alphabets representing significant difference (P<0.05)



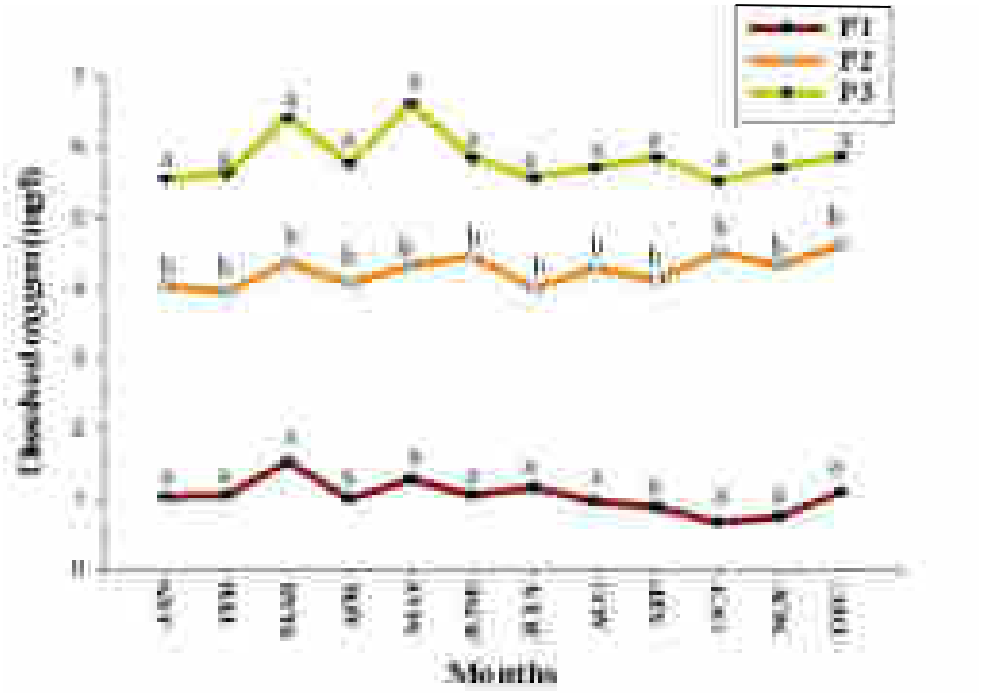
The pH of the water sample ranged between 6.9 and 8.1. The minimum pH 7.8 was noticed in June and September, 2017 and the maximum pH range 8.1 was noticed in January, 2017 at station P1. In station P2 minimum 7.4 pH was observed in the month of June, September and December, 2017. The maximum 7.8 pH was recorded in the month of January, 2017. Sampling station P3, showed a minimum pH of 6.9 and was recorded in March, 2017 whereas the maximum 7.4 pH range was noticed in August and October, 2017 (Fig. 8).

Fig 9. Total suspended solids of study ponds at Kumbakonam during January – December 2016; Each Point represents mean ± SE of 3 samples; a posteriori Holm-Sidak comparison of study ponds are shown with different alphabets representing significant difference (P<0.05)



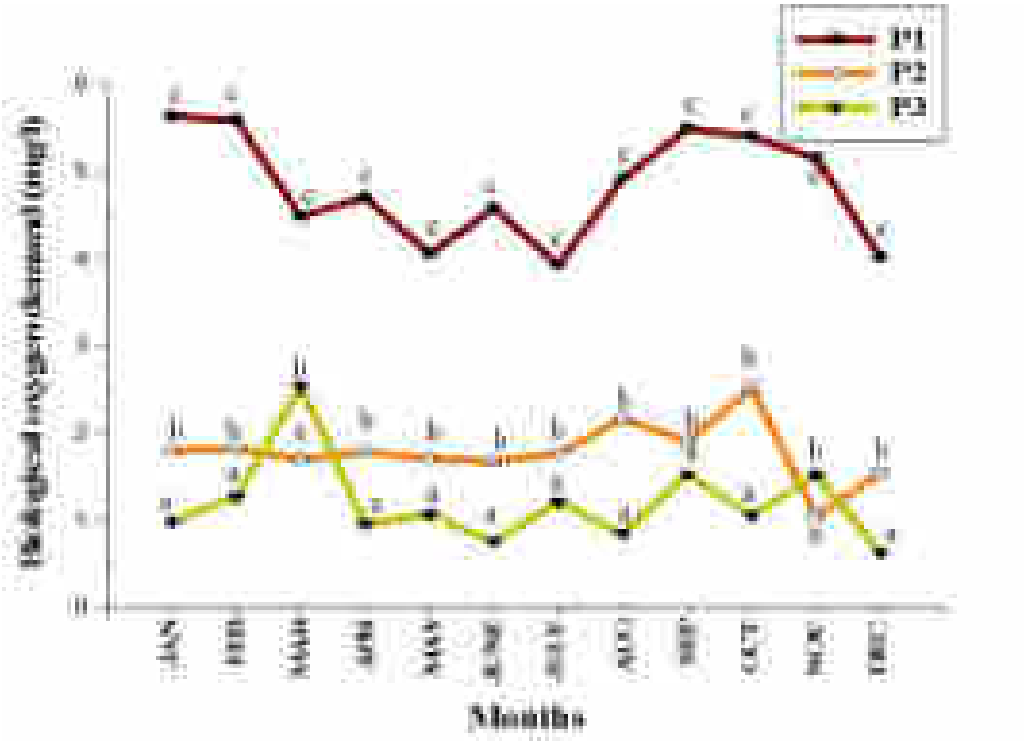
The Total suspended solids wavered from 28.30 to 112.60mg/l. The highest (112.60mg/l) value of TSS found in the month of October, 2017 and the lowest value (70.30mg/l) was found in December, 2017 at station P1. In Station P2 the minimum value (35.20mg/l) obtained in December, 2017 and the maximum value (69.80mg/l) during March, 2017. In Station P2 the minimum TSS 28.30mg/l was recorded in December, 2017 and the maximum 44.70mg/l was recorded in August, 2017. (Fig.9).

Fig 10. Dissolved oxygen of study ponds at Kumbakonam during January – December 2016; Each Point represents mean ± SE of 3 samples; a posteriori Holm-Sidak comparison of study ponds are shown with different alphabets representing significant difference (P<0.05)



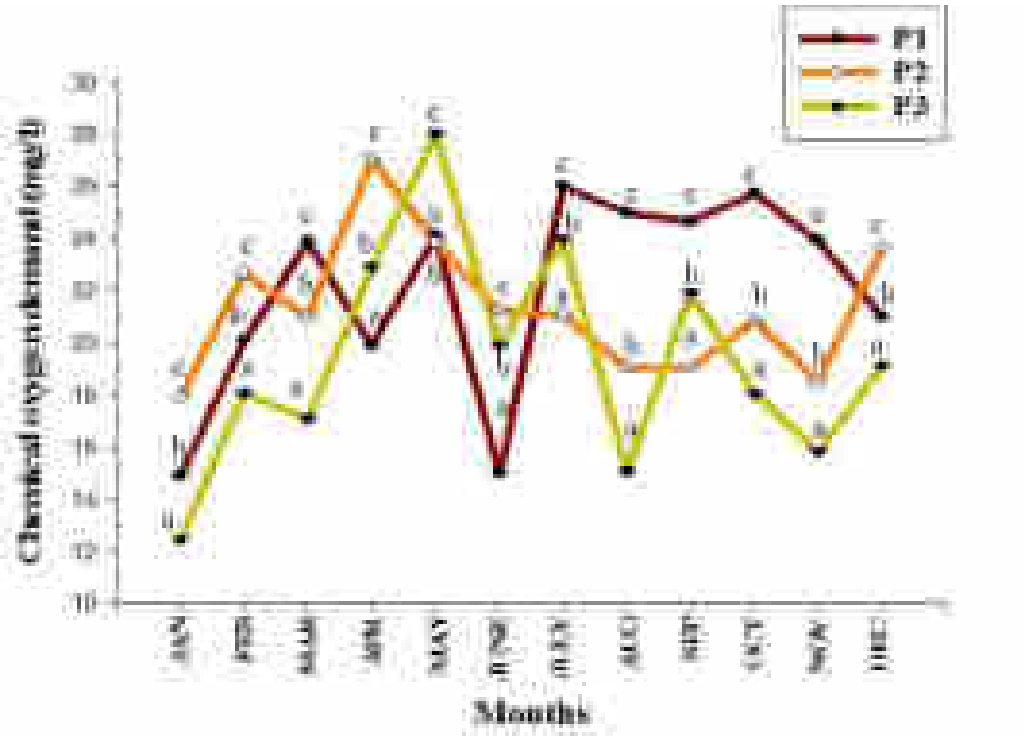
The quality of water is ascertained based on the Dissolved oxygen value. The dissolved oxygen demand value ranged from 0.665 mg/l to 6.632 mg/l in the study area. The minimum value (0.665 mg/l) concentration of DO was recorded during the month of October, 2017 and the maximum (1.532mg/l) was recorded during the month of March, 2017 at P1 station. In P2 station, the maximum (4.624mg/l) DO was noted in the month of December, 2017 and the minimum (3.954mg/l) level of DO was noted in the month of February, 2017. Least level (5.524mg/l) of DO was found in October, 2017 and the highest level (6.632mg/l) of DO was found in P3 station during the month of May, 2017. (Fig.10).

Fig 11. Biological oxygen demand of study ponds at Kumbakonam during January December 2016; Each Point represents mean ± SE of 3 samples; a posteriori Holm-Sidak comparison of study ponds are shown with different alphabets representing significant difference (P<0.05)



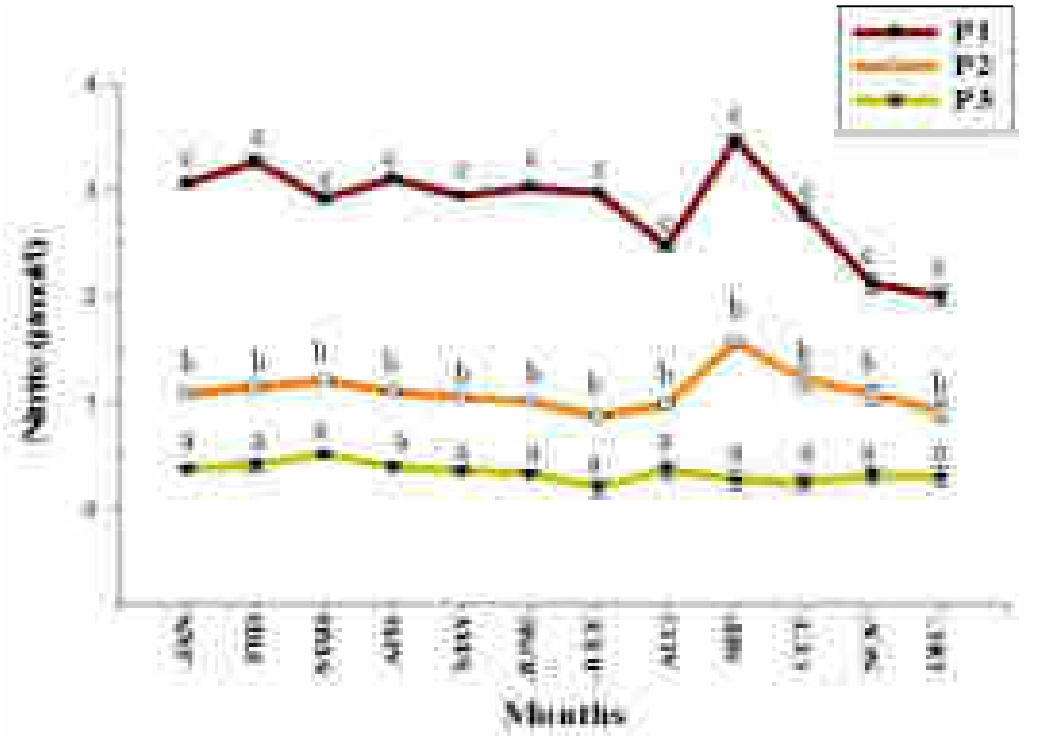
The biological oxygen demand value ranged from 1.045 mg/l to 5.624mg/l among stations P1, P2 and P3. The minimum value (3.916mg/l) of BOD was recorded during the month of July, 2017 and the maximum (5.624mg/l) was recorded during the month of January, 2017 in P1 station. In P2 station, the maximum (2.508mg/l) BOD was noted in the month of October, 2017 and the minimum (1.045mg/l) level of BOD was noted in the month of November, 2017. Least level (2.008mg/l) of BOD was found in December, 2017 and the highest level (5.0704mg/l) of BOD was found in P3 station during the month of March, 2017. (Fig.11).

Fig 12. Chemical oxygen demand of study ponds at Kumbakonam during January – December 2016; Each Point represents mean ± SE of 3 samples; a posteriori Holm-Sidak comparison of study ponds are shown with different alphabets representing significant difference (P<0.05)



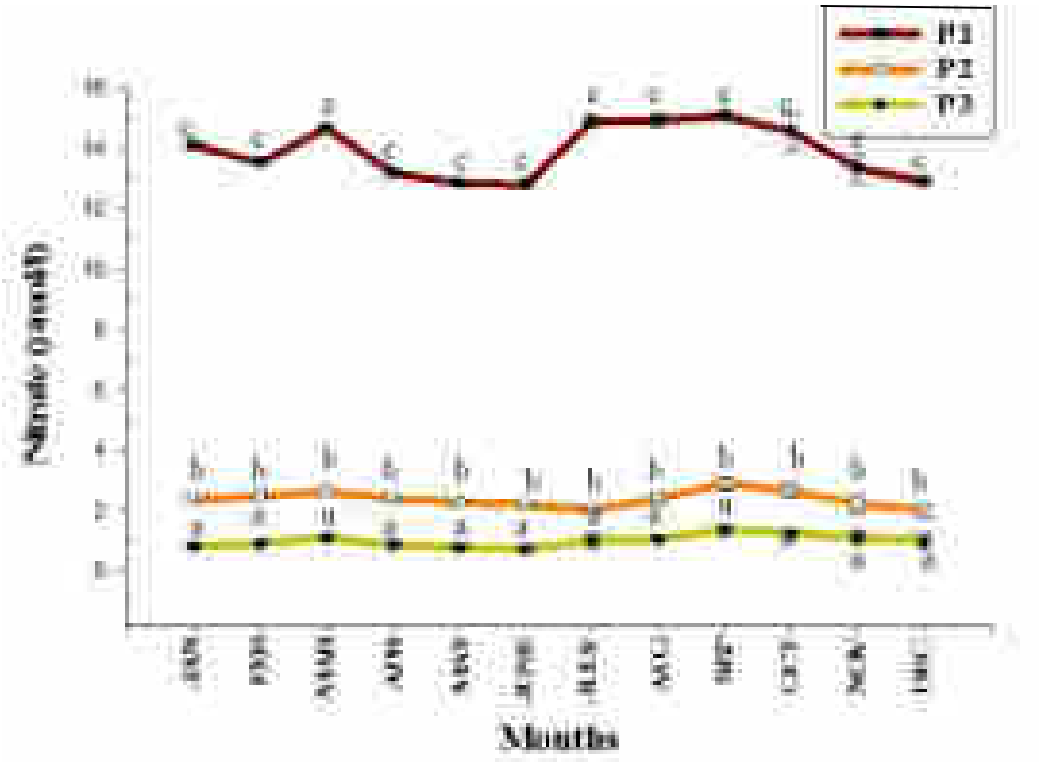
The chemical oxygen demand range was recorded as 12.467 to 28.005mg/l. the minimum (14.906mg/l) chemical oxygen demand was noticed in the P1 station during the month of January, 2017 and the maximum level (26.003mg/l) of COD was found in P1 station during the month of July, 2017. The maximum (27.061mg/l) value of COD was recorded in P2 station during April month and the minimum value (17.893 mg/l) was recorded at P2 station in the month of January, 2017. The value for COD is maximum (28.005 mg/l) at P3 station during the month of May, 2017 and the minimum (12.467mg/l) was observed during the month of January, 2017. (Fig.12).

Fig 13. Nitrite content of Study Ponds at Kumbakonam during January – December 2016; Each Point represents mean ± SE of 3 samples; a posteriori Holm-Sidak comparison of study ponds are shown with different alphabets representing significant difference (P<0.05)



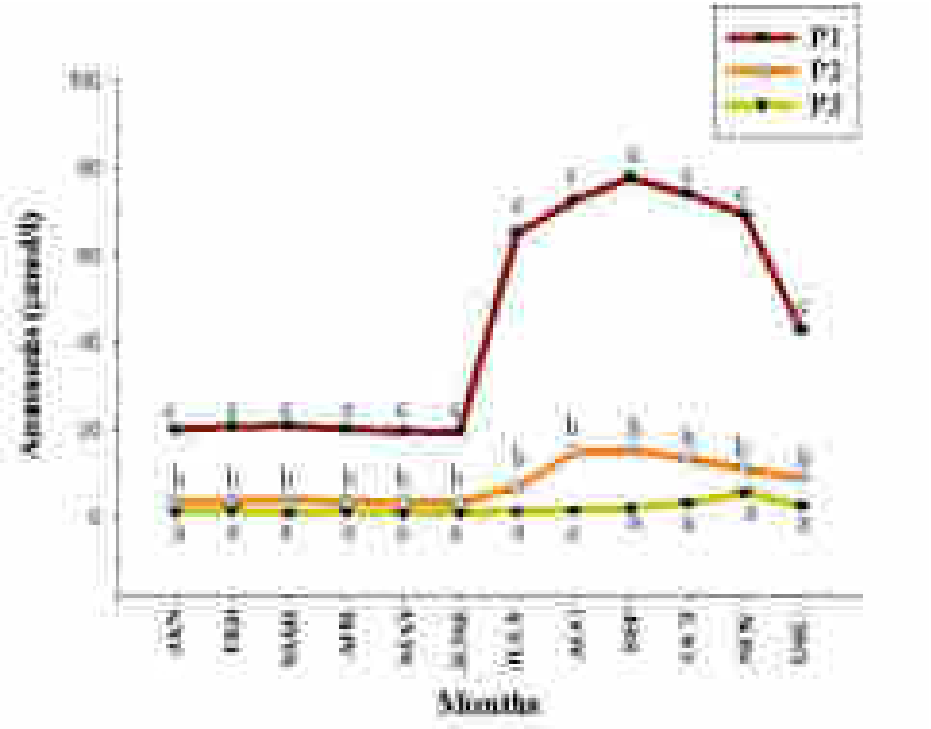
For Nitrite, the minimum value (2.006 µmol/l) was recorded in P1 station during the month of December and the maximum (3.471 µmol/l) was recorded in the month of September, 2017 at P1 station. In station P2, the maximum (1.573 µmol/l) was recorded in the month of September and the minimum (0.875 µmol/l) was recorded in the month of July, 2017. The minimum (0.202 µmol/l) of nitrite was observed in P3 station during the month of July, 2017 and the maximum (0.514 µmol/l) was observed during the month of March, 2017 in P3 station. (Fig.13).

Fig 14. Nitrate content of study ponds at Kumbakonam during January – December 2016; Each Point represents mean ± SE of 3 samples; a posteriori Holm-Sidak comparison of study ponds are shown with different alphabets representing significant difference (P<0.05)



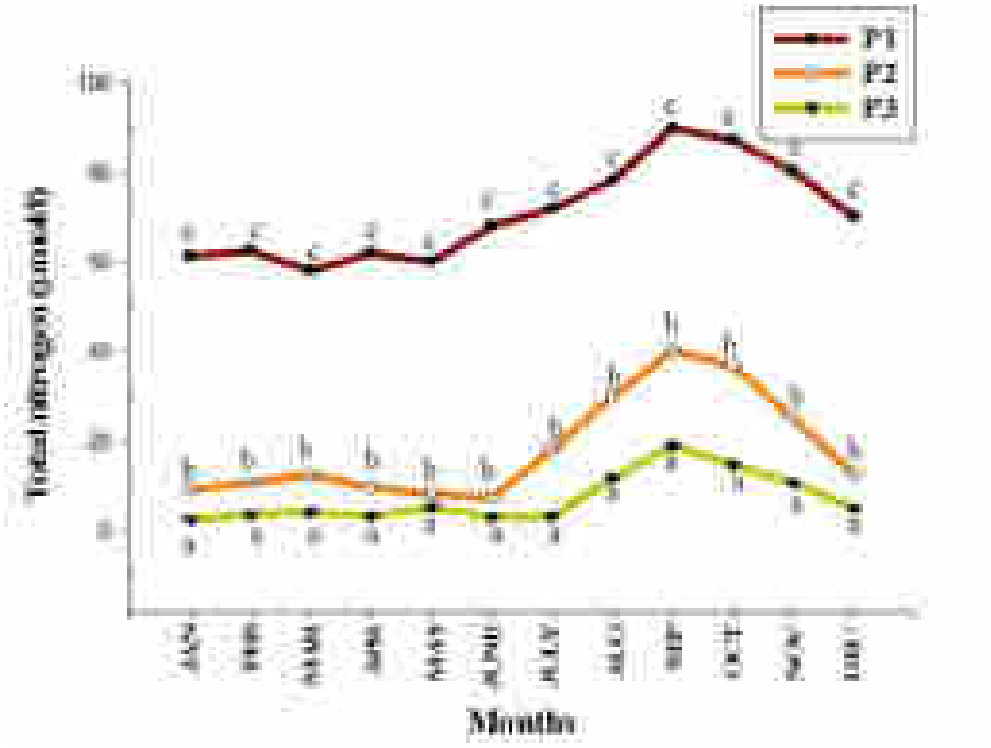
During the study period the minimal (12.796µmol/l) content of NO_3 was noted at P1 station in June, 2017 and the maximal (15.072 µmol/l) content of NO_3 was noted in the month of September, 2017. The maximum range (2.896 µmol/l) of NO_3 was noticed in P2 station during the month of September, 2017 and the minimum (2.006 µmol/l) was noticed at P2 station in the month of December, 2017. In station P3, the maximum (1.346 µmol/l) limit of NO_3 was observed in September, 2017 and the minimum (0.725 µmol/l) limit of NO_3 was observed in June, 2017 (Fig.14).

Fig 15. Ammonia content of study ponds at Kumbakonam during January – December 2016; Each Point represents mean ± SE of 3 samples; a posteriori Holm-Sidak comparison of study ponds are shown with different alphabets representing significant difference (P<0.05)



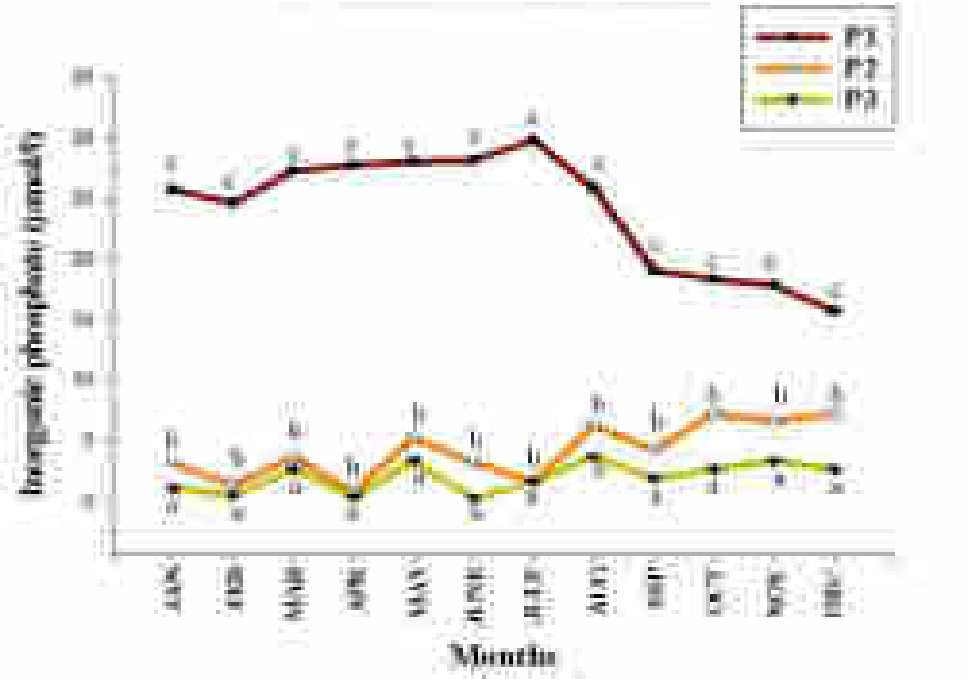
The concentration of ammonia fluctuates from 0.914 to77.71µmol/l. The minimum (19.599 µmol/l) amount of NH_4 was observed in June, 2017 at P1 station and the maximum value (77.716 µmol/l) was observed at P1 station in September, 2017. In station P2, the maximum (15.285 µmol/l) of NH_4 was noted during the month of September, 2017 and the minimum (3.332 µmol/l) was noted during the month of June, 2017. The maximum (5.612 µmol/l) of NH_4 content was recorded in November, 2017 at P3 station and the minimum content (0.941 µmol/l) was recorded in May, 2017 at P3 station (Fig.15).

Fig 16. Total Nitrogen of study ponds at Kumbakonam during January – December 2016; Each Point represents mean ± SE of 3 samples; a posteriori Holm-Sidak comparison of study ponds are shown with different alphabets representing significant difference (P<0.05)



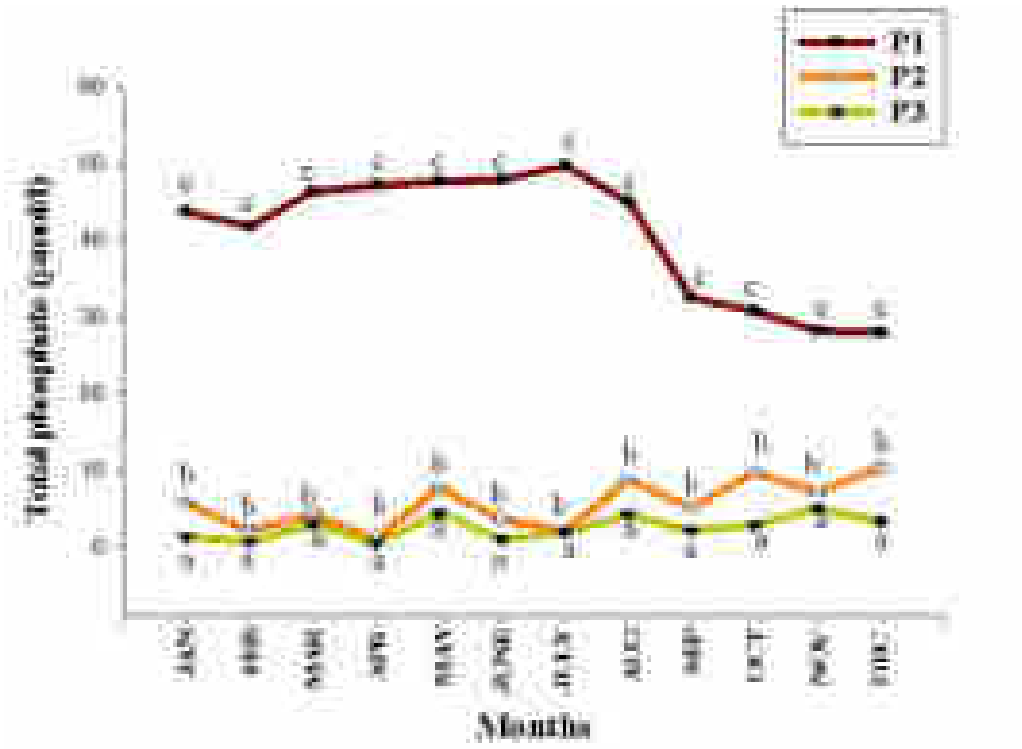
In the present investigation total nitrogen was recorded in the range between 2.562 to 89.991µmol/l. In P1 station the minimum (58.081 µmol/l) was noticed during the month of March, 2017 and the maximum (89.991 µmol/l) of TN was noticed during the month of September, 2017. The higher value (40.305 µmol/l) of TN was observed in the month of September, 2017 at P2 station and the lower value (7.306 µmol/l) of TN was observed in the month of June, 2017 at P2 station. In P3 station, least level (2. 562 µmol/l) of TN was recorded during the month of January, 2017 and the highest value (18.980 µmol/l) was recorded during the month of September, 2017. (Fig.16).

Fig 17. Inorganic Phosphate of study ponds at Kumbakonam during January – December 2016; Each Point represents mean ± SE of 3 samples; a posteriori Holm-Sidak comparison of study ponds are shown with different alphabets representing significant difference (P<0.05)



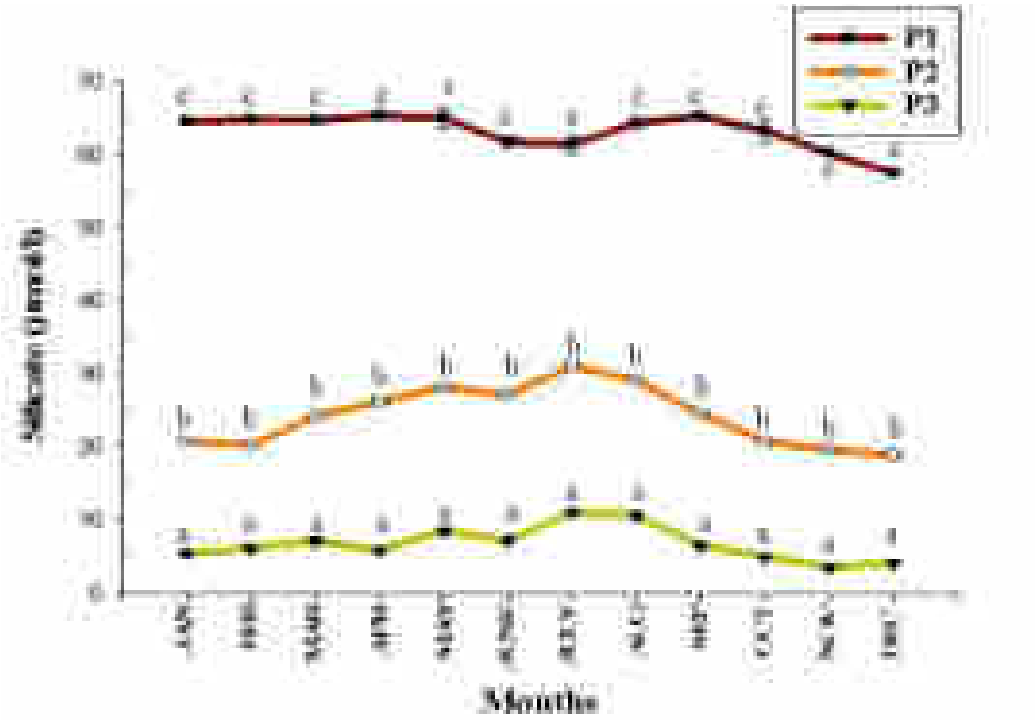
The range of inorganic phosphate ion content was 0.158 to 29.788µmol/l. The maximum (29.788µmol/l) concentration of IP was noted in the month of July, 2017 and the minimum (15.629µmol/l) IP was noted in the month of December, 2017 at P1 station. In P2 station, the minimum (0.685µmol/l) range of IP was observed during the month of April, 2017 and the maximum (7.152µmol/l) of IP was observed during the month of October and December, 2017. The highest level (3.630µmol/l) of IP was recorded in August, 2017 at P3 station and the lowest value (0.158µmol/l) was recorded in June, 2017 at P3 station. (Fig. 17).

Fig 18. Total Phosphate of study ponds at Kumbakonam during January – December 2016; Each Point represents mean ± SE of 3 samples; a posteriori Holm-Sidak comparison of study ponds are shown with different alphabets representing significant difference (P<0.05)



In this study, the minimum (27.904µmol/l) total phosphate was observed in P1 station during the month of December, 2017 and the maximum (49.711µmol/l) was observed during the month of July, 2017. In P2 station the maximum (10.260 µmol/l) of TP was recorded during the month of December, 2017 and minimum (1.025 µmol/l) level was recorded during the month of April, 2017. The month of April, 2017 had the minimal level of TP (0.413 µmol/l) at P3 station and the maximal level (4.956 µmol/l) of TP was noted during the month of November at P3 station. (Fig.18).

Fig 19. Silicate of study ponds at Kumbakonam during January – December 2016; Each Point represents mean ± SE of 3 samples; a posteriori Holm-Sidak comparison of study ponds are shown with different alphabets representing significant difference (P<0.05)



The monthly variations of silicate among the ponds were found to be in the range from 3.510 to 66.233µmol/l. The highest level (66.233 µmol/l) of silicate in the month of May and the lower (57.420 µmol/l) in the month of December, 2017 at P1 station was recorded. At Station P2, the minimum (18.864 µmol/l) limit was recorded during the month of December, 2017 and the maximum (31.050 µmol/l) limit was recorded during the month of July, 2017. The maximum (11.056 µmol/l) of Sio_3 was observed in July, 2017 and the minimum (3.510 µmol/l) of Sio_3 was observed during the month of November, 2017 at P3 station. (Fig.19).

Table 1. Pearson correlation matrix of physiochemical parameters of pond waters

	Tem	*Salin*	*pH*	*DO*	*TSS*	*BOD*	*COD*	*NO_2*	*NO_3*	*NH_4*	*TN*	*IP*	*TP*	*SiO_3*
Tem	1													
Salin	0.464	1												
pH	0.161	-0.110	1											
DO	-0.140	0.407	-0.577	1										
TSS	0.523	0.356	0.311	-0.334	1									
BOD	-0.193	-0.204	0.231	**-0.322**	0.588	1								
COD	**0.788***	0.428	0.142	**-0.361**	0.389	-0.356	1							
NO_2	0.080	-0.094	-0.255	-0.076	0.446	0.629	-0.050	1						
NO_3	0.241	0.243	0.235	-0.256	**0.745***	**0.730***	-0.046	0.403	1					
NH_4	0.362	0.374	0.408	-0.417	0.428	0.212	0.309	-0.254	0.607	1				
TN	0.325	0.312	0.321	-0.398	0.504	0.420	0.255	0.019	0.669	**0.932*****	1			
IP	0.491	0.442	-0.004	0.489	0.241	-0.231	0.161	-0.097	-0.031	-0.213	-0.317	1		
TP	0.464	0.296	-0.069	0.448	0.165	-0.267	0.169	0.014	-0.116	-0.397	-0.479	**0.958*****	1	
SiO_3	0.729*	0.510	-0.124	0.175	0.563	0.008	0.479	0.409	0.269	-0.076	-0.064	**0.749***	**0.784***	1

Correlation significant level 0.05*, 0.01**, 0.001***

Pearson's correlation coefficient(r) were made to find the physico-chemical parameters like water temperature, salinity, pH, TDS, BOD, COD, DO, NO_2, NO_3, NH_4, TN, IP, TP and SiO3.The degree of correlation between the parameters is represented in Table. 1. The water temperature was positively correlated with COD (r=0.788) and Sio3 (r=0.729) at P<0.05 level of significance. Salinity showed a negative correlation with BOD (r=-0.204) and NO2 (r=-0.094). The pH was negative correlation with DO (r=-0.577) and NO_2 (r=-0.225), IP (r=-0.04), TP (r=-0.069) and SiO_3 (r=-0.124). DO has negative correlation with BOD (r=-0.322) and COD (r=-0.361). TSS have strongly positive correlation with NO_3 (r=0.745) with a statistical significance level of 0.05. BOD expressed a positive correlation with NO_3 (r=0.730) at P<0.05 level. NH4 represented a strong correlation with TN (r=0.932) with a statistical significance at 0.001 P value. IP exhibited a high positive correlation with TP (r=0.958) and SiO_3 (r=0.749) at P<0.001 and P<0.05 level of significance, respectively. TP also expressed a positive correlation with SiO_3 (r=0.784) with statistical significance of P<0.05 level.

Zooplankton diversity

According to the present study, 27 numbers of different zooplankton species were recorded at Mathi pond, 14 species at Pidari pond and 17 species at Sei Pond belonging to the groups of Copepoda, Cladocera, Rotifera and Ostrocoda. The maximum number of species observed belong to the family Copepoda in Mathi Pond (P1) and the minimum number of species recorded belong to groups of Cladocera and Rotifera at Pidari Pond (P2) were shown in Table 2. Diversity indices, richness and evenness for three stations calculated.

Table 2 showed the distribution of different zooplankton species in three different study ponds. It reveals that a total of 12 species comprising of 4 Copepods, 2

Cladocerans, 3 Rotifers and 3 Ostracods are found to be common in all the three ponds. Similarly 9 species are found unique in Mathi Pond. They are *Macrocylops albidus, Moina brachiata, Leydigia leydigia, Asplanchana brightwelli, Asplanchana intermedia*. However, the other two ponds do not possess such unique species.

Table 2: List of zooplankton recorded in the Mathi Pond, Pidari Pond and Sei Pond at Kumbakonam, Thanjavur District, Tamilnadu, India during the months of one year (January - December, 2017)

S. No	Species	Mathi Pond	Pidari Pond	Sei Pond
	COPEPODA			
1	*Sinodiaptomus sarsi*	+	+	+
2	*Mesocyclops leuckarti*	+	-	+
3	*Mesocyclops pehepiensis*	+	-	+
4	*Thermocyclops hyalinus*	+	+	+
5	*Cyclops vernalis*	+	+	+
6	*Eucyclops speratus*	+	+	+
7	*Macrocylops edax*	+	+	-
8	*Macrocylops albidus*	+	-	-
	CLADOCERA			
9	*Diaphanosoma sarsi*	+	+	+
10	*Daphnia magna*	+	+	+
11	*Ceriodaphnia cornuta*	+	-	-
12	*Moina micrura*	+	+	+
13	*Moina brachiate*	+	-	-
14	*Leydigia leydigia*	+	-	-
	ROTIFERA			
15	*Asplanchna intermedia*	+	-	-
16	*Asplanchna brightwelli*	+	-	-
17	*Brachionus rubens*	+	-	+
18	*Brachionus rotundiformis*	+	+	+
19	*Brachionus calyciflorus*	+	+	+
20	*Brachionus caudatus pesonatus*	+	+	+
	OSTROCODA			
21	*Cypris protubera*	+	+	+
22	*Cypris decaryi*	+		+
23	*Eucypris bispinosa*	+	+	+
24	*Cyprinotus nudus*	+	+	+
25	*Heterocypris dentatomarginaturs*	+	-	-
26	*Heterocypris claus*	+	-	-
27	*Prinocypris glacialis*	+	-	-

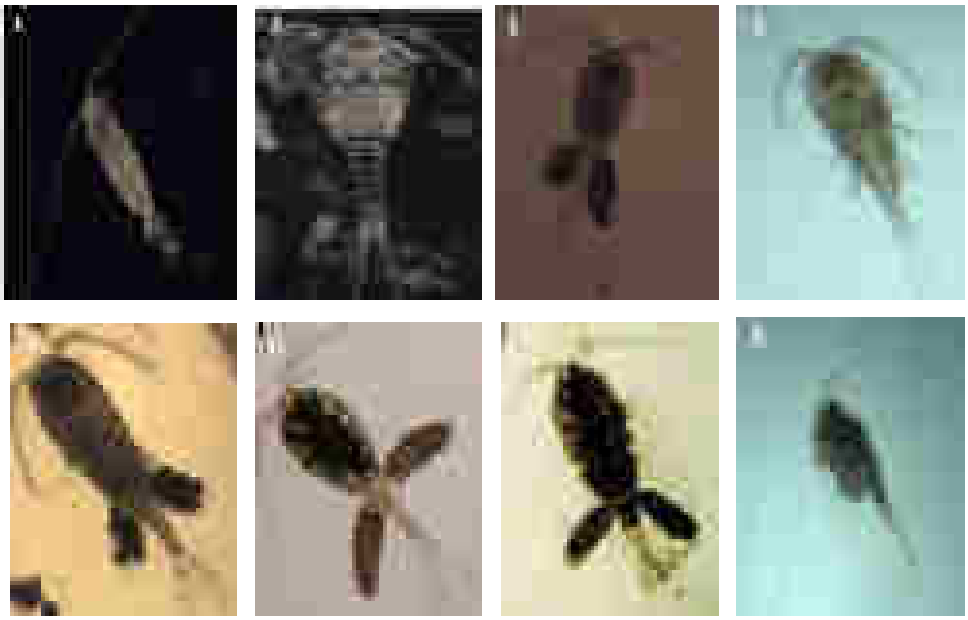


Plate 15. COPEPOD SPECIES

1. ***Heliodiaptomus viduus 2. Eucyclops speratus*** **3.** ***Cyclops vernalis 4. Thermocyclops pehpeiensis 5. Mesocyclops leuckarti 6. Mesocyclops edax 7. Mesocyclops pehepiensis 8. Macrocyclops albidus***

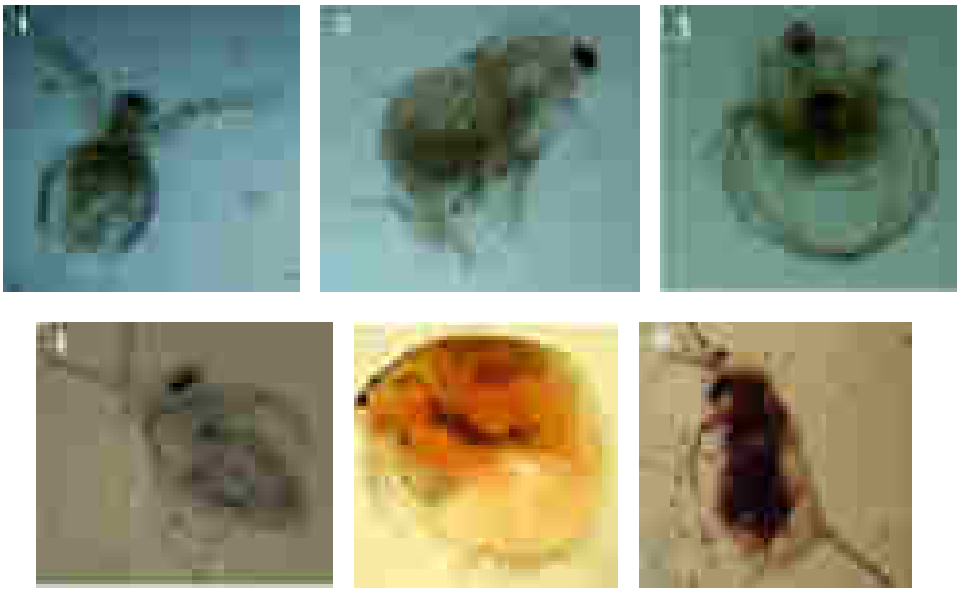


Plate 16. CLADOCERA SPECIES

1. ***Diaphanosoma sarsi 2. Moina brachiate*** **3.** ***Ceriodaphnia carnuta 4. Moina micrura 5. Leydigia leydigia 6. Daphnia magna***

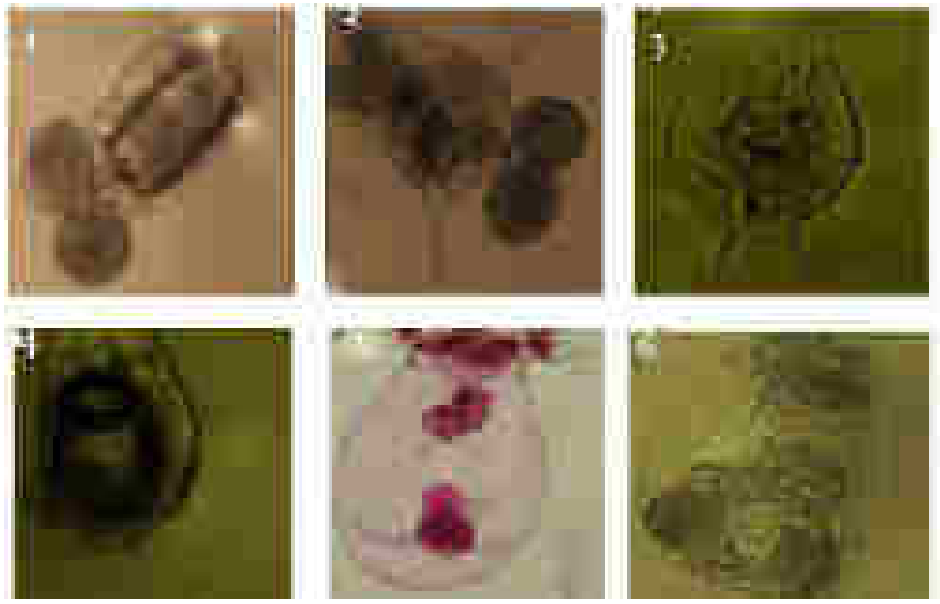


Plate 17. ROTIFERA SPECIES

1. ***Brachionus roundiformi 2. Brachionus calyciflorus*** **3.** ***Brachionus diversicornis 4. Brachionus caudatus personatus 5. Brachionus rubens 6. Aslanchna brightwelli 7. Asplanchna intermedia***

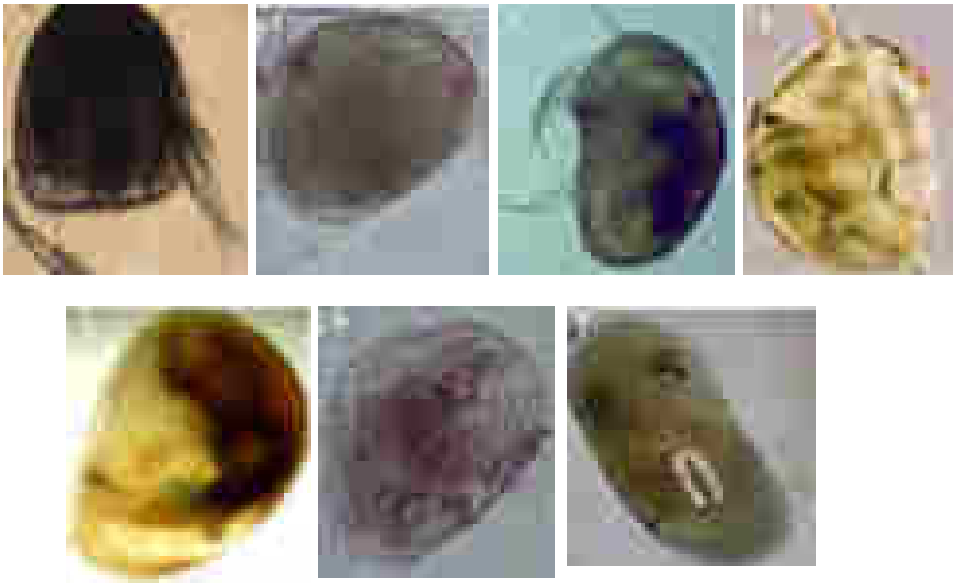


Plate 18. OSTROCODA SPECIES

1. Cypris protubera 2. Eucypris bispinosa 3. Cypris dearyi 4. Heterocypris dentatomarginatus 5. Candona candida 6. Cyprinotus nudus 7. Prionocypris glacialis

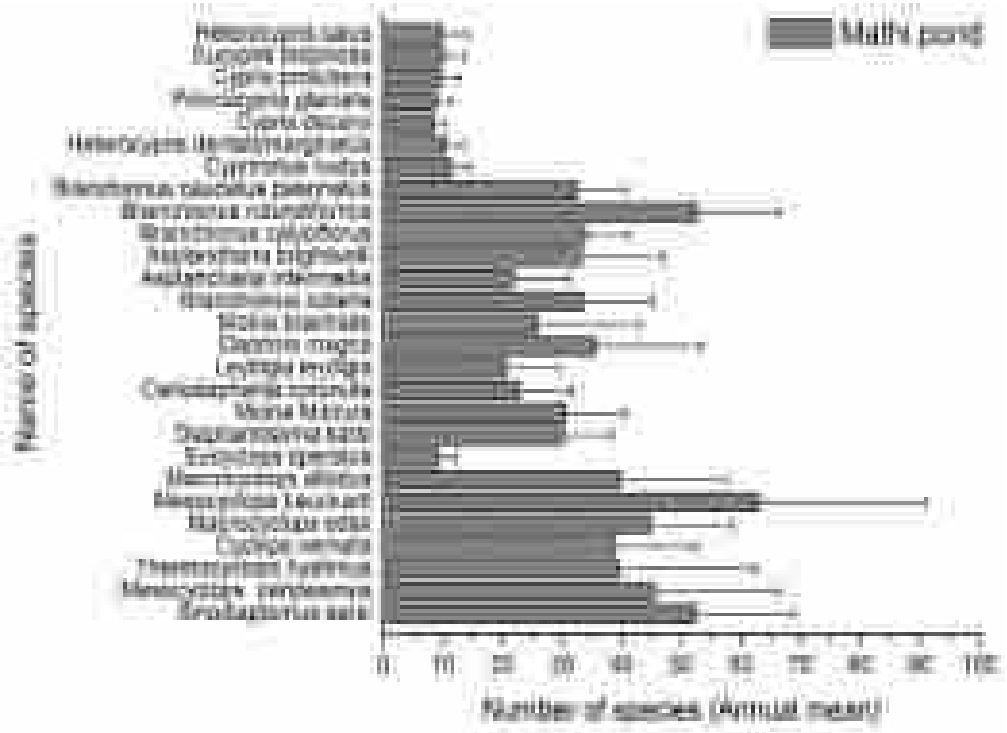


Fig 20. Zooplankton observed in Mathi Pond

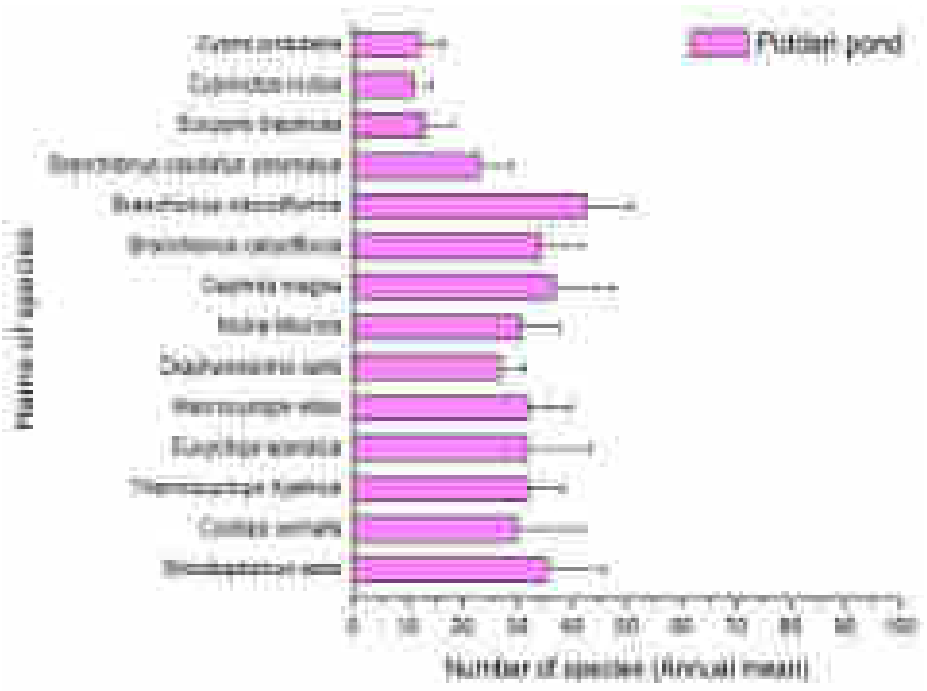


Fig 21. Zooplankton observed in Pidari Pond

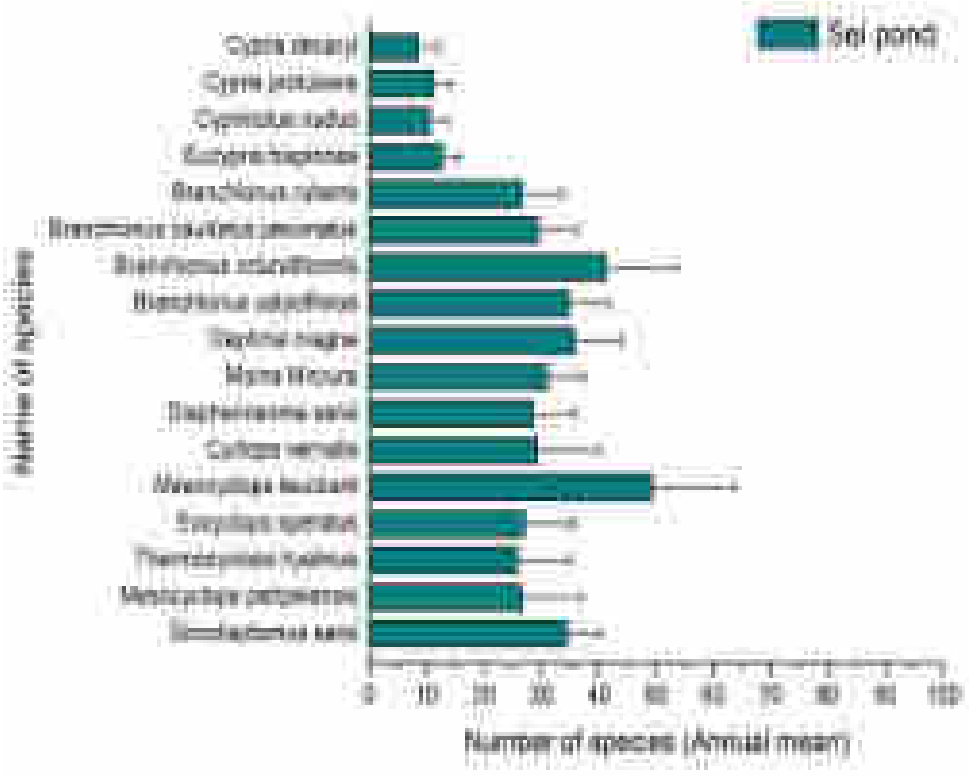


Fig 22. Zooplankton observed in sei Pond

Fig 20 exhibits the quantitiy of different zooplankton species observed in Mathi Pond. According to this, the copepod *Mesocclops leuckarti* is found dominant succeeded by the rotifer *Brachionus rotundiformis*.

Fig 21 displays the number of different zooplankton species observed in Pidari Pond throughout the year. It shows at rotifers *Brachionus rotundiformis* is the dominant species succeeded by the copepod *Sinodiaptomus sarsi.*

Fig 22 clearly shows that in Sei Pond out of the 17 species observed, *Mesocyclops leuckarti* is the dominant species followed by *Brachionus rotundiformis* and *Sinodiaptomus sarsi.*

Shannon – Wiener index – H' (log^2)

The Shannon-Wiener H' (log^2) diversity index calculations showed that the minimum (3.088) diversity was found on September and the maximum (3.214)

individuals were found in month of June in P1 station. In stationP2, the maximum (2.591) was recorded in September month and the minimum (2.536) was recorded in the month of January. In station P3, the minimum (2.671) species diversity was observed during the month of February and the maximum (2.754) diversity was observed during the month of June. Among the ponds, P2 exhibits minimum diversity (2.536 to 2.591) and station 1 had maximum diversity of 3.088 to 3.214. Table. 3.

Table: 3 Diversity, richness and evenness of Zooplankton species in pond waters

	Diversity index			Species Richness			Species Evenness		
Month	**P1**	**P2**	**P3**	**P1**	**P2**	**P3**	**P1**	**P2**	**P3**
Jan	3.120	2.536	2.739	3.995	2.146	2.545	0.936	0.961	0.967
Feb	3.132	2.544	2.671	4.011	2.184	2.430	0.940	0.964	0.963
Mar	3.109	2.544	2.732	4.251	2.297	2.708	0.933	0.964	0.964
Apr	3.187	2.553	2.742	4.241	2.283	2.723	0.956	0.968	0.968
May	3.208	2.539	2.739	4.268	2.260	2.742	0.963	0.962	0.967
June	3.214	2.561	2.754	4.277	2.251	2.774	0.965	0.970	0.972
July	3.201	2.558	2.741	4.214	2.235	2.698	0.961	0.969	0.968
Aug	3.174	2.578	2.735	4.117	2.188	2.608	0.952	0.977	0.965
Sep	3.088	2.591	2.749	3.941	2.125	2.545	0.927	0.982	0.97
Oct	3.122	2.570	2.733	3.956	2.110	2.514	0.937	0.974	0.965
Nov	3.102	2.570	2.739	3.846	2.097	2.517	0.931	0.974	0.967
Dec	3.124	2.543	2.737	3.833	2.081	2.486	0.938	0.964	0.966

Margalef richness (d) index

In P1 station, the Margalef richness (d) varied between 3.833 to 4.277. Minimum (3.833) was recorded in the month of December and the maximum (4.277) was recorded in the month of June. In P2 station, highest level (2.297) of species richness was noted during the month of March and the lowest level (2.081) of richness was noted during the month of December. In station P3, higher level (2.774) of richness was observed in the month of June and the lower level (2.430) of richness was observed in the month of February. Table. 3.

Pielou's evenness index (J')

Species evenness was in the range of 0.927 to 0.965 during the month of June and September respectively at P1 station. Highest (0.982) evenness index was recorded at P2 station during the month of September and the lowest (0.961) species evenness was recorded during the month of January. In station P3, the maximum (0.972) evenness was found on the month of June and the minimum (0.963) was found on the month of February, 2017 (Table. 3).

Zooplankton population density

Fig 23. shows the Zooplankton population density in Mathi pond throughout the year. It has maximum zooplankton density during December with 1147 nos./l and minimum during the month of June 552 nos./l out the study period, it was noted that copepods occupied the maximum population followed by rotifers and minimum of ostracods**.**

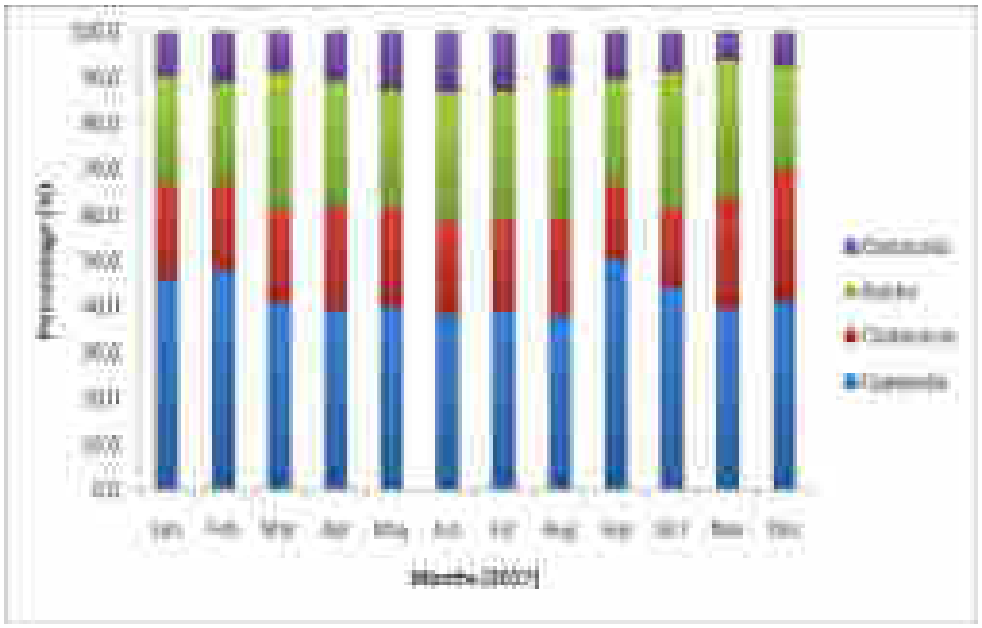


Fig 23. Population density in Mathi Pond

Fig 24. reveals the zooplankton density during the study period in Pidari pond**.** The total number of zooplankton was highest during December (516nos./l) and lowest during the month of March (287nos./l). In Pidari Pond also the same composition of zooplankton was observed in the order of Copepoda > Rotifera > Cladocera > Ostracoda.

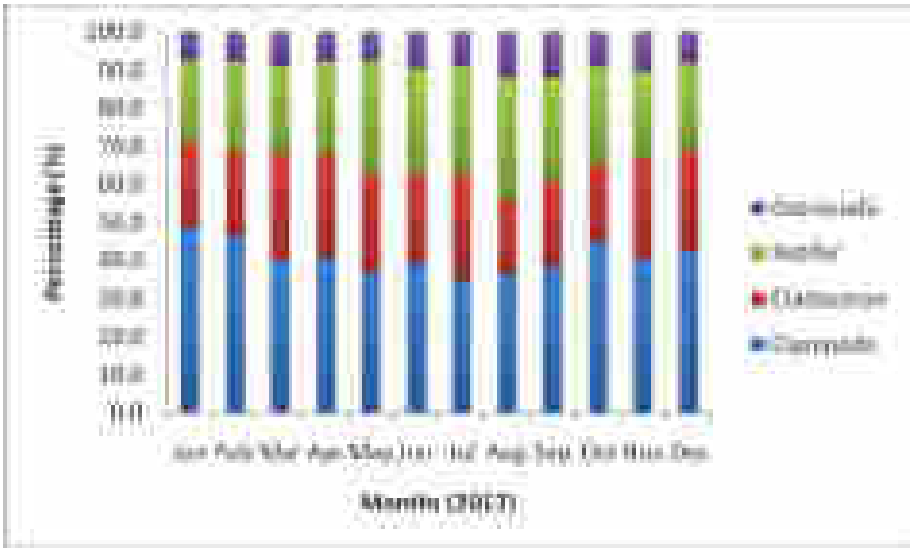


Fig 24. Population density in Pidari Pond

The Sei pond zooplankton density pattern during the study period was depicted in Fig. 25. It shows that the zooplankton population was maximum in the month of December with 624 nos./l and minimum in the month of June with 320 nos./l Here also the copepods are the dominating community and ostracods the least.

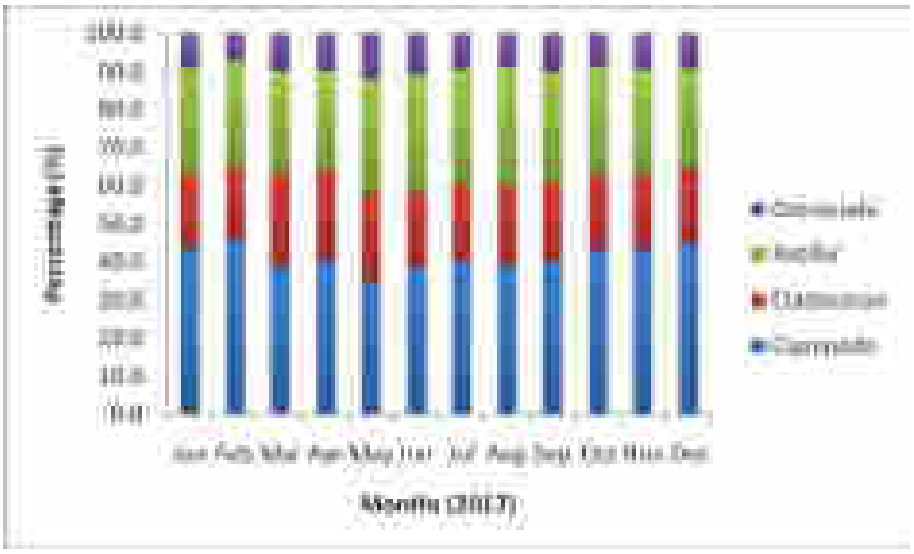


Fig 25. Population density in Sei Pond

Cluster Analysis

In order to define the similarity and dissimilarity, the data acquired from the three different stations were applied for cluster analysis. The dendrogram from cluster analysis revealed that the stations Mathi pond, Pidari pond and Sei pond were formed at the higher and lower level of similarities during the period of one year (Jan-Dec, 2017). In Mathi pond, cluster has generated into 5 groups. Group 1 and 2 includes the month of August and March, whereas the third group includes the month of May, July, April and June. Group 4 formed a single cluster during the month of September. Group 5 includes the month of January, February, October and November- December. Maximum (96%) zooplankton diversity was seen during the month of April to July and the minimum (86%) was exhibited in the month of September (Fig. 26).

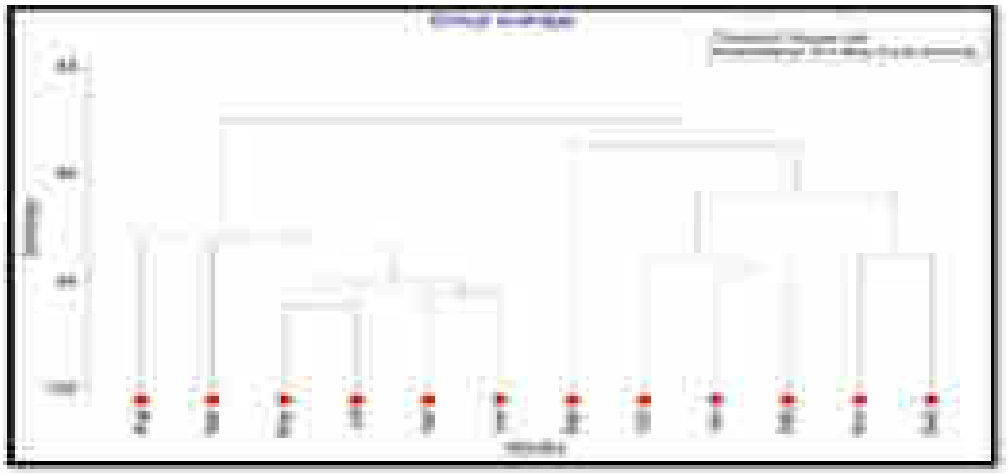


Fig 26. Dendrogram of zooplankton diversity in Mathi Pond

In Pidari pond, cluster generated into 3 groups. Group 1 includes September-December and January- February. Group 2 formed a single cluster during the month of August. Group 3 includes in the month of March to July. In cluster analysis of Pidari pond, the month of August showed a lower (92%) similarity of zooplankton diversity and the higher (96.5%) level of species similarity in the month of October and December (Fig. 27).

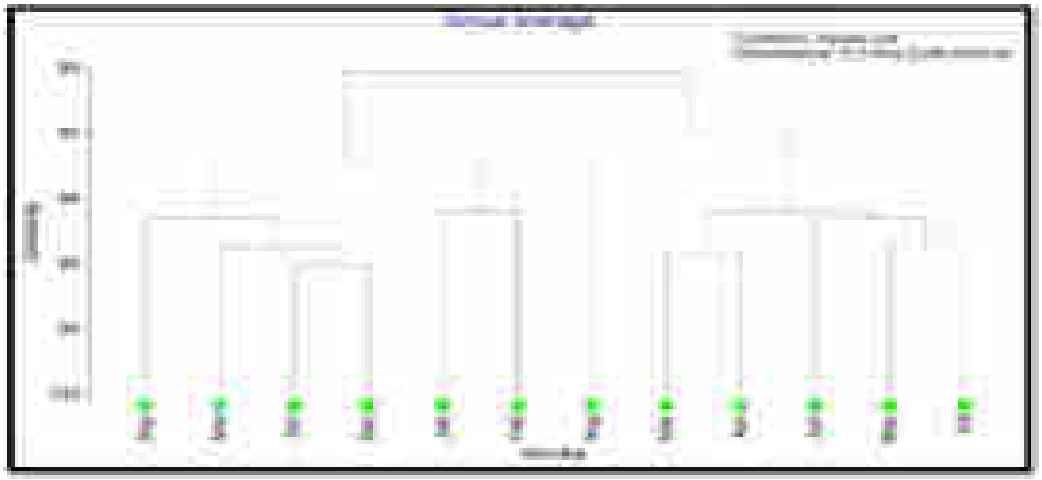


Fig 27. Dendrogram of zooplankton diversity in Pidari Pond

In Sei pond, 3 clusters were observed during the period of January to December, 2017. In months of May, June, July, March and April showed similar association and grouped into single cluster. Similarly, the month of January, October, November, December and August, September has group formation and exhibited maximum similarity was observed during the month of January and November. In contrast, the month of February showed a very minimum similarity (Fig. 28).

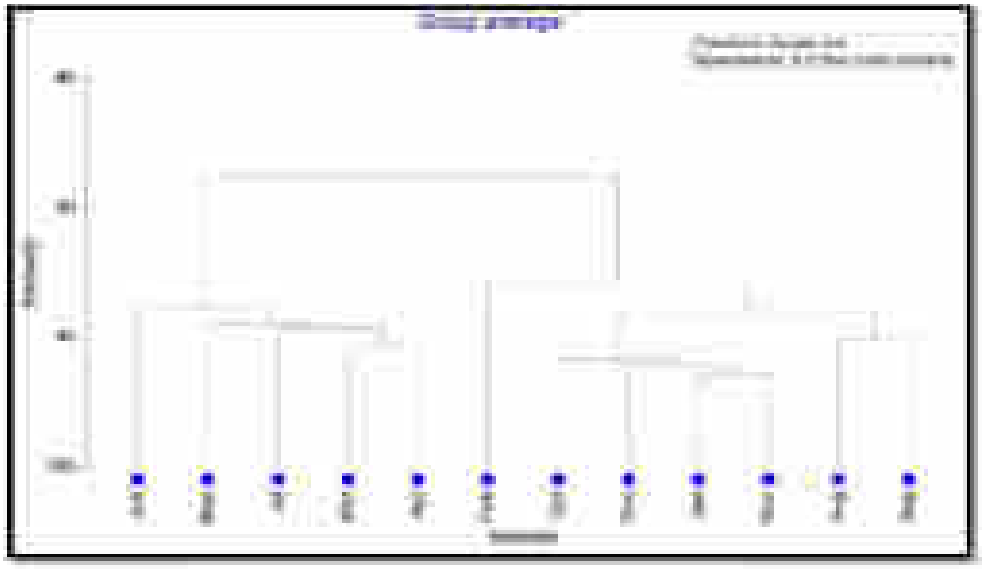


Fig 28. Dendrogram of zooplankton diversity in Sei Pond

Molecular identification

The freshwater zooplankton species *Mesocyclops leuckarti* and *Sinodiaptomus sarsi* were found to be maximum in numbers and hence studied for their morphological observations. The molecular characteristics of COI gene of these species are described below.

Morphological descriptions

Mesocyclops leuckarti is easily distinguished from other cyclopoid copepods by the last pair of legs (P5). The antennae consist of 17 segments and the

setae on the outer edge of the tail is attached near the middle. In *M. leuckarti* the length of the lateral seta of the furca is comparatively longer and about half as long as the inner setae. The colour is generally pale yellow, with a more or less distinct bluish green tinge.

Sinodiaptomous sarsi has first antennae at least half the length of the body and biramous second antennae. Caudal setae equal or unequal in length. 1 egg sac, carried medially. First antennae reach from near end of metasome to near end of caudal setae, female 23–25 segments. Leg 5 similar to other legs, or modified, basal portion 2 segments, endopod present or not, 3 segmented or modified.

Molecular phylogeny inferred from 18S rRNA gene

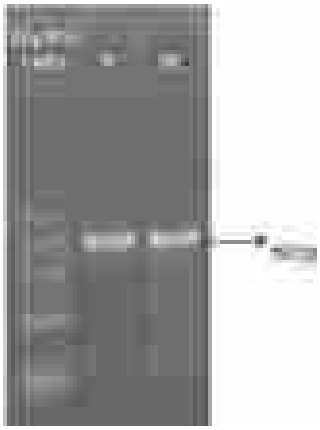


Plate. 19. Electrophoresis profile of amplified PCR product of 500bp filament of the 18S rRNA sequences

The amplified products of two freshwater zooplankton species were sequenced and the processed sequences are deposited in NCBI and accession numbers were obtained for the two particular species. The accession number assigned for *Mesocyclops leuckarti* and *Sinodiaptomus sarsi,* several other 18S rRNA sequences of the genus *Mesocyclops* and *Sinodiaptomus* species were retrieved from NCBI via Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi)

for constructing the phylogenetic tree. The results obtained were recorded with *Mesocyclops leuckarti* and *Sinodiaptomus sarsi.*

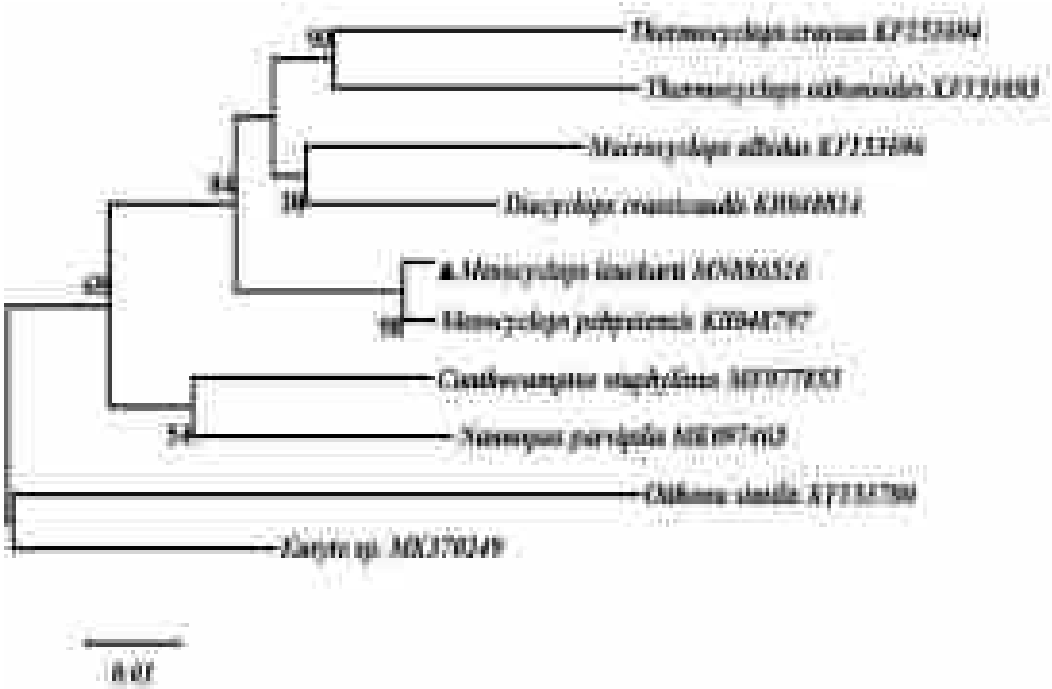


Fig 29. Construction of inter-specific phylogenetic tree of 18S rRNA gene from closely related sequences obtained from different organism using MEGA 6.0 (*Mesocyclops leuckarti*) MN886516

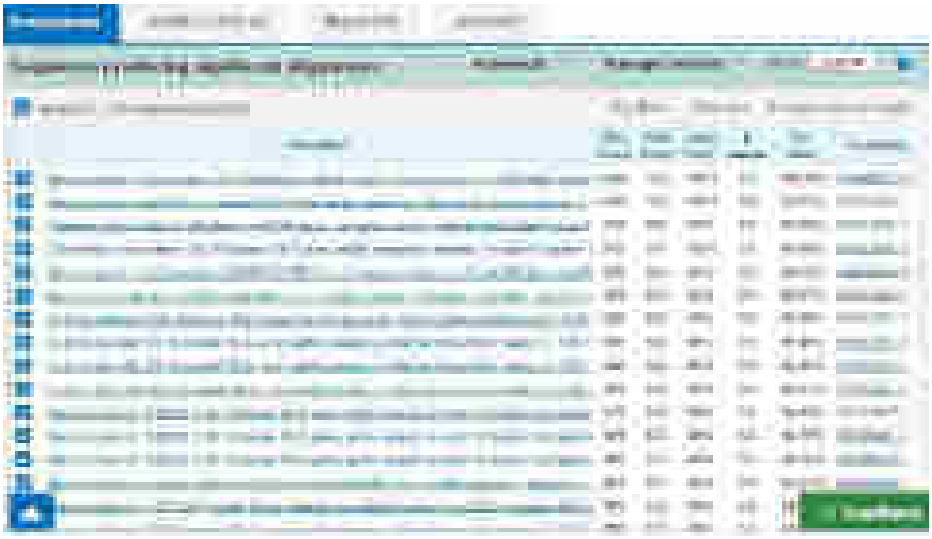


Fig 30. Similarity comparison of *Mesocyclops leuckarti*

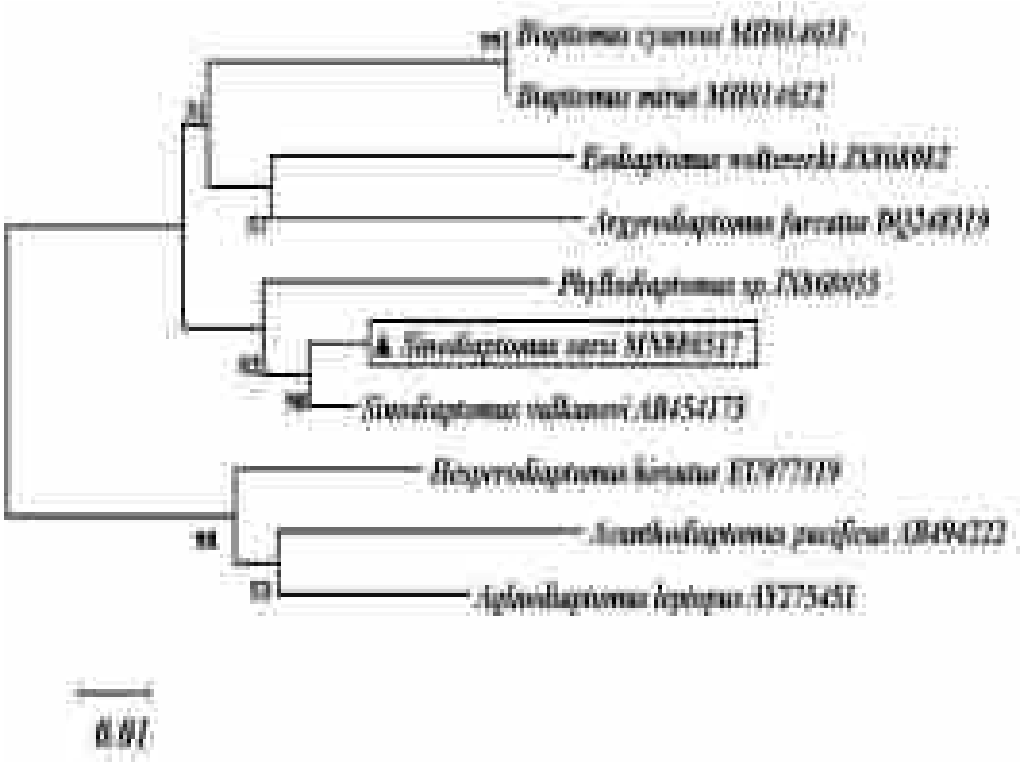


Fig 31. Construction of inter-specific phylogenetic tree of 18S rRNA gene from closely related sequences obtained from different organism using MEGA 6.0 (*Sinodiaptomus sarsi*) MN886517

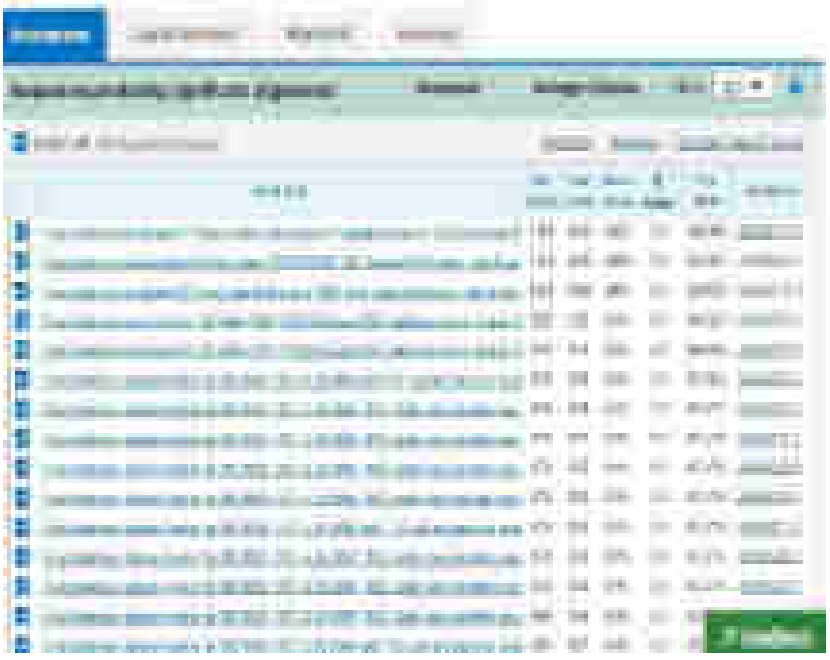


Fig 32. Similarity comparison of *Sinodiaptomus sarsi*

The phylogenetic tree was constructed to test the efficacy of 18S rRNA genes among related species of *Mesocyclops leuckarti* (MN886516) and *Sinodiaptomus sarsi* (MN886517) to its species level. *Sinodiaptomus sarsi* showed 90% similarity with *Sinodiaptomus valkanovi.* Further the blast-n results of 18S rRNA genes of the two copepods of interest are depicted in Fig. 29 and Fig. 31. The results confirmed that the MN886516 as *Mesocyclops leuckarti* and MN886517 as *Sinodiaptomus sarsi. Mesocyclops leuckarti* 18S rRNA gene sequence with an accession number MN886516 was used to ascertain the phylogenetic relationship among different related species. The constructed phylogenetic tree showed 98% similarity with *M. pehpeiensis*. Bar-coded sequence of *Euryte* sp. was used as an out group in the phylogenetic tree. The results were also confirmed with the manual morphological characteristics analysis.

Survivability of Copepods in culture under laboratory conditions

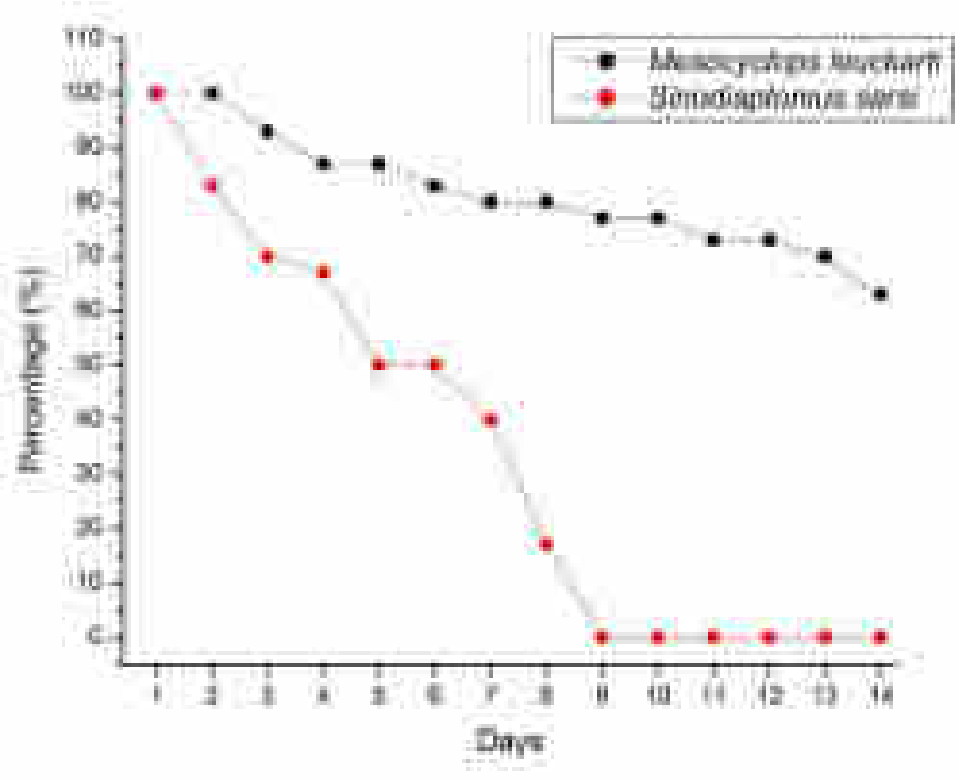


Fig 33. Survivability of different copepod species in culture

Fig 33. Shows the *Mesocyclops leuckarti* has mor survivability than *Sinodiaptomus sarsi*. 70% of the Mesocyclops population in culture survived till the end of the experimental period while the other species *Sinodiaptomus sarsi* could not survive under laboratory conditions. The population start declining from day one and 0% survival was observed on 9th day of the experiment.

Toxicity of Cow Urine Distillate for *Mesocyclops leuckarti*

Table 4. Lethal concentration LC50 on *Mesocyclops leuckarti*

Concentration ppm	**Mortality**	**LC_{50} 95% (LCL-UCL)**	**LC_{90} 95% (LCL-UCL)**	**R^2**
0.2	10			
0.4	20			
0.6	50	0.57 (0.45-0.74)	0.91 (0.89-0.94)	0.979
0.8	70			
1.0	100			

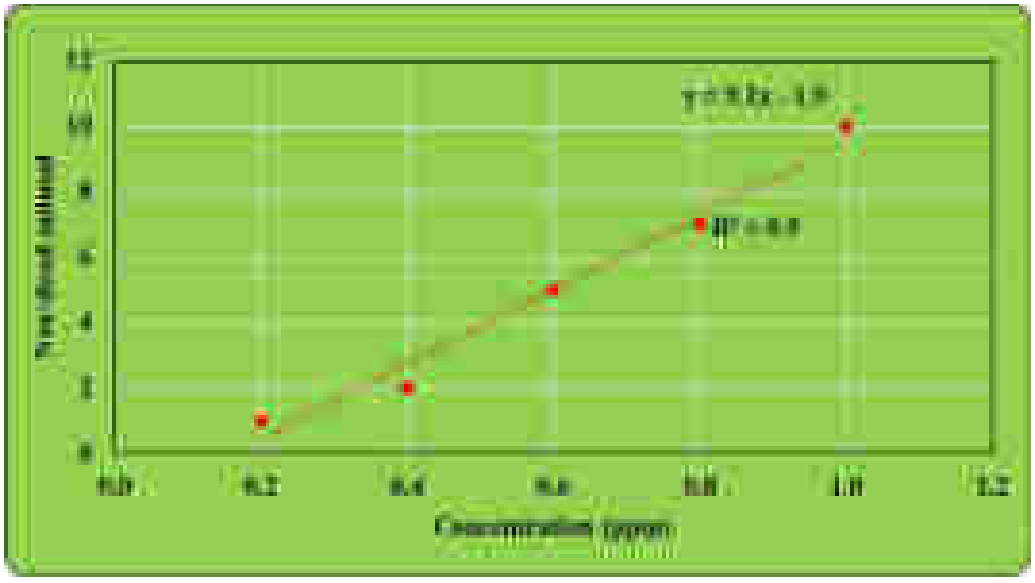


Fig. 34 Slopes and intercepts of regression line for CUD toxicity on *Mesocyclops leuckarti*

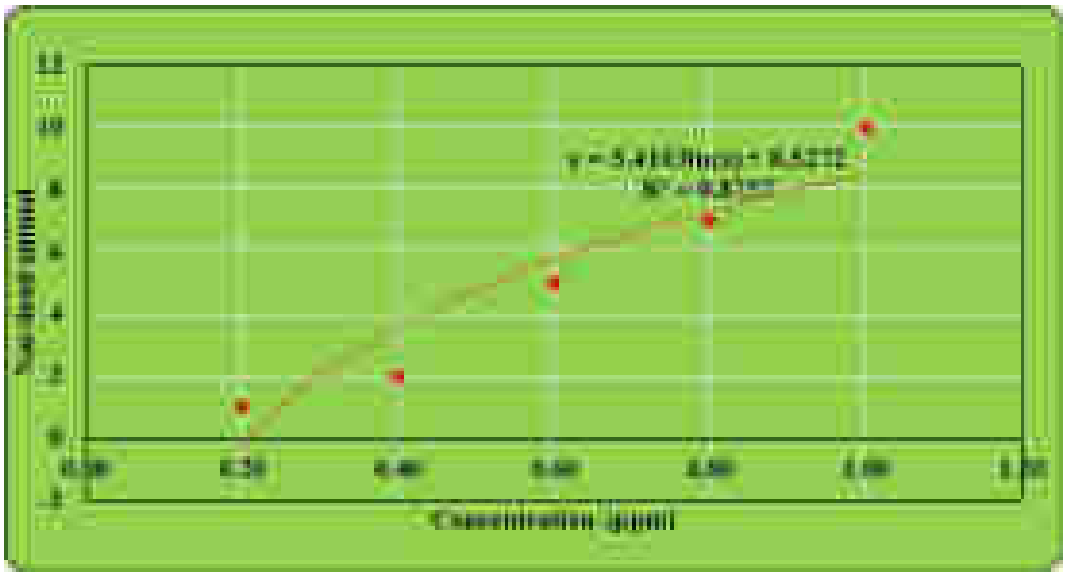


Fig. 35 Slopes and intercepts of logarithmic regression line for CUD toxicity on *Mesocyclops leuckarti*

In the present study toxicity effects of CUD have been studied on *Mesocyclops leuckarti.* Experiments were conducted in duplicate. Test animals were collected from stock zooplankton culture. Only actively swimming animals were selected for the experiments. Five concentrations of CUD ranging from 0.2%, 0.4%, 0.6%, 0.8 and 1% were used. Twenty animals were used for each of the 5 concentrations and control. For each concentration animals were divided into two groups of 10 individuals (in duplicate) in 500 ml beaker. Adequate controls were also maintained in duplicate. Animals observed to be weak immediately after transferring were replaced. Test animals were starved and experiment beakers were not aerated during the tests.

Each test casted 24 hrs and mortality was noted every 4, 8, 12, 16, 20 and 24hr. Death of individuals was determined from absence of movement even when prodded gently. A computerised probit analysis was carried out for toxicity test. Probit regression against log concentration were made for CUD-Zooplankton

combination and LC50 values were calculated. Derived LC50 values, slopes and intercepts of the individual regression lines are presented in Table 4. LC50 values show significant differences in tolerance between the difference concentration of cow urine distillate when compared to control. Tests with cow urine distillate for copepod showed steeper slopes indicating large change in percentage mortality for small variations in concentrations. Cow urine distillate was highly toxic to copepod at 1% (100% mortality) than at 0.6% (50% mortality), with a significantly lower LC50 value (95% confidence limits) of 0.57 (0.45-0.74) µg CUD/l, compared to control.

Population density of copepod culture

Based on the results of Chapter I, Chapter II and Chapter III, the copepod *Mesocyclops leuckarti* has been identified as the dominant and suitable species for further studies. Hence it has been selected for culture under lab conditions.

Survival

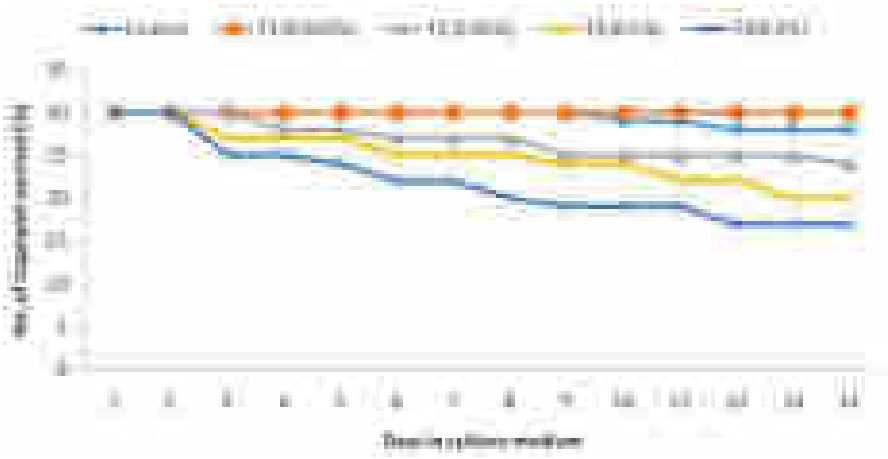


Fig 36. Effect of CUD on survival of *Mesocyclops leuckarti* in culture

The different concentrations of cow urine distillate had made a notable impact on the survival percentage of copepods. At the end of an experimental period of 14 days, the trial T1 (0.025%) resulted with the highest survival rate of 100%. The Control treatment resulted in 93% survival, whereas the lowest percentage of survival

was recorded in trial T4 (0.2%) with 56%. The other trials T2 (0.05%) and T3 (0.1%) resulted with a survival rate of 80% and 66% respectively (Fig. 36). In trial T4, the survival rate of copepods tends to decrease from the beginning itself.

Nauplii production rate (NPR)

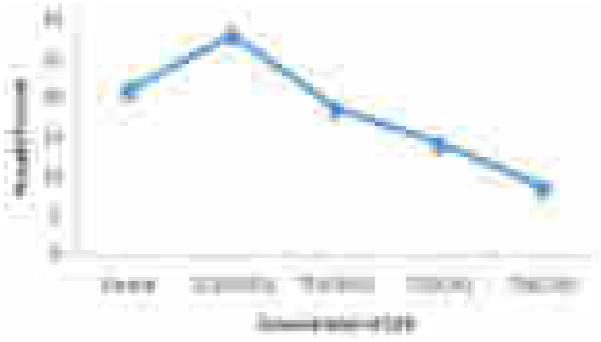


Fig 37. Effect of CUD on Nauplii Production Rate of *Mesocyclops leuckarti*

Likewise, in survival test, the nauplii producing capacity of the copepod was considerably affected by the concentration of CUD treatments. An average of 28 naupllii were produced by a female in a single brooding in T1, which is highest among the trials, followed by Control which produced 21 nauplii per female. The trial T2 produced an average of 18.6 nauplii per female. The trials T3 and T4 produced the lesser number of nauplii at an average of 14.2 and 8.6 nauplii per female respectively (Fig. 37). The rate of production of nauplii was relatively lower with the higher concentration of treatments.

Population density

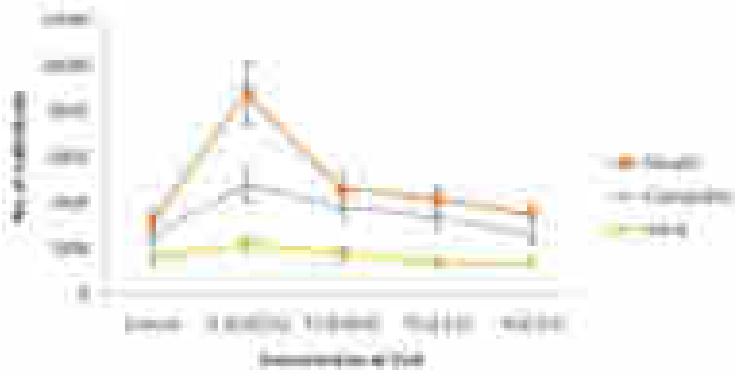
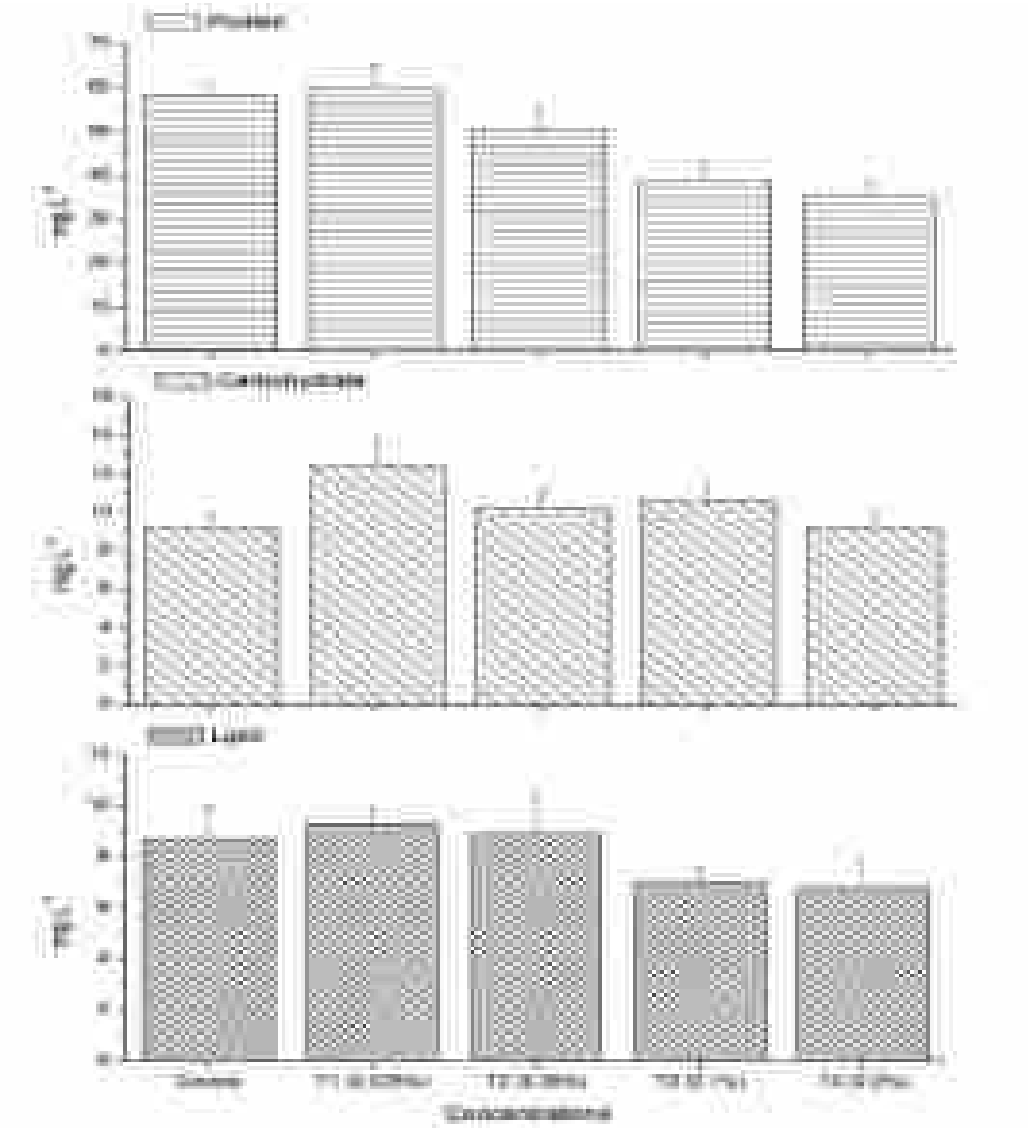


Fig 38. Effect of CUD on Population density of *Mesocyclops leuckarti* in culture

The average total number of organisms (inclusion of nauplii, copepodites and adults) at the end of an experimental period of 30 days is shown in Fig. 38. The highest number of *Mesocyclops leuckarti* density was obtained in T1 with a total of 15800 ind/l, which comprised of 8800 nauplii, 4800 copepodite and 2200 adults. The lowest total of copepod density were recorded in T4, with an average of 7500 ind/l, which comprises of 3500 nauplii, 2600 copepodites and 1400 adults.

Biochemical composition



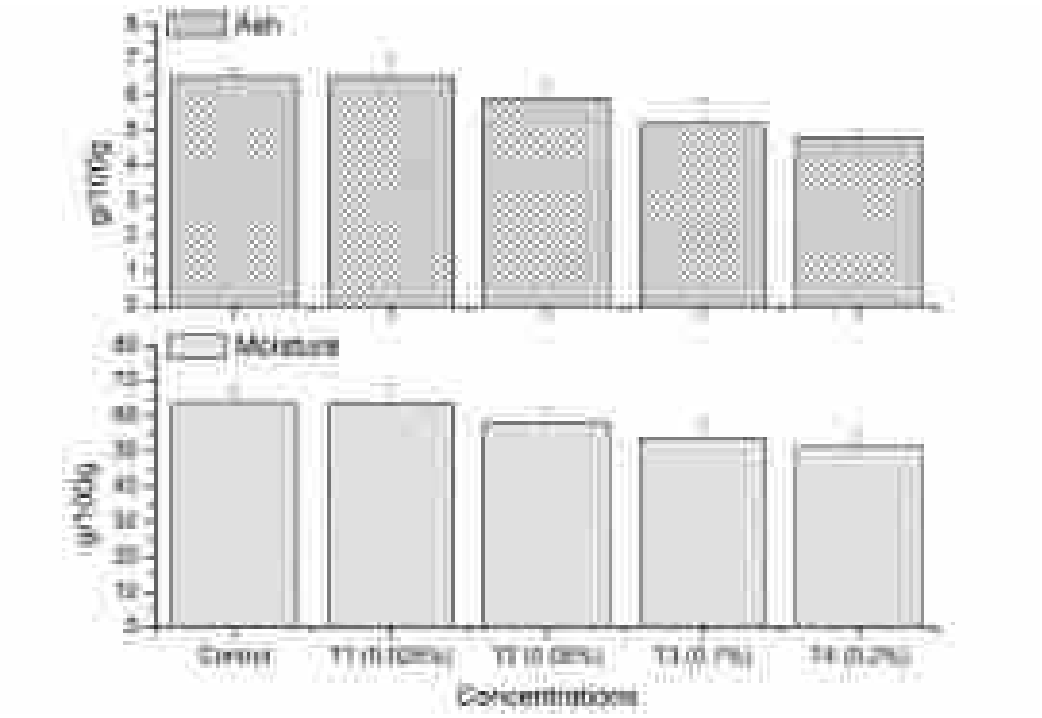


Fig 39. Effect of CUD on the biochemical composition of *Mesocyclops leuckarti*

The results on biochemical composition of freshwater copepod *Mesocyclops leuckarti* cultured under different trials were shown in Fig. 39. The difference in biochemical composition between the trials showed greater variations. The highest protein content of copepod *M. Leuckarti* was observed in T1 group with 60.17%, followed by Control (58%), whereas the lowest protein content was observed in T4 with 35.3%. The carbohydrate content was higher in T1 (12.4%) followed by T3 (10.6%) and the lowest value was obtained in both Control and T4 (9.2%). The total lipid content of *M. Leuckarti* was higher in T1 (9.3%) followed by T2 (8.9%) whereas the lowest value was observed in T4 (6.8%). Among the trials, the moisture content was higher in control treatment with 85.41% followed by T1 (63.76%). The lowest value of moisture was obtained in T4 with 51.26%. The ash content was higher in control treatment (6.53%) followed by T1 (6.46%) and the lowest ash content was observed in T4 (4.8%).

Immunological studies

Hemocyte cell identification

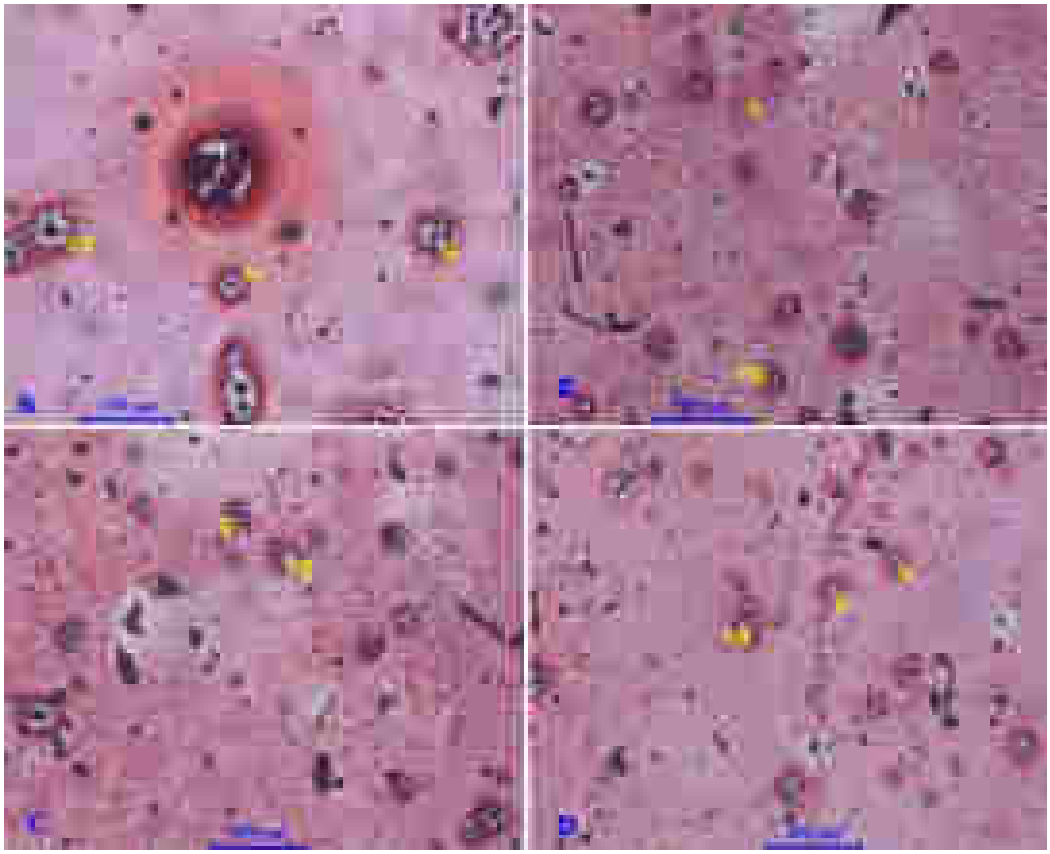


Plate 20. Hemocyte cell identification from hemolymph of *Mesocylops leukarti* by using phase contrast microscope

Freshly packed hemocytes stained with eosin were examined by light microscopy (LM) to determine cell size, cell shape and presence of granules under a magnification of 100X. A calibrated micrometer was used for cell measurements. Our results suggest that hemocytes of cyclopoid copepod are composed of three major groups, hyaline cells, semi granulocytes and granulocytes, which have distinct morphological differences. Most investigators have historically recognized these three categories and separated those using morphological criteria. Based upon results with Eosin staining, the *Mesocyclops leuckarti* hemocyte cells were classified according to

size, shape and presence or absence of cytoplasmic granules. (i) Hyaline cells: Cells with no evidence of cytoplasmic granules. These are found in abundant (Figure 40). (ii) Semi granulocytes: Cells with the variable number of cytoplasmic granules. (iii) Granulocytes cells with a great number of cytoplasmic granules.

Isolation of High and Low molecular proteins

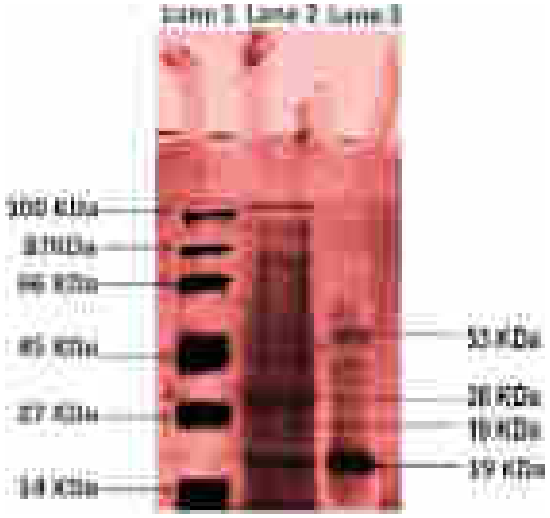


Plate 21. Gel electrophoresis *Mesocyclops leuckarti* protein molecules

Antibacterial Activity of High and Low molecular weight proteins of *Mesocyclops leuckarti*

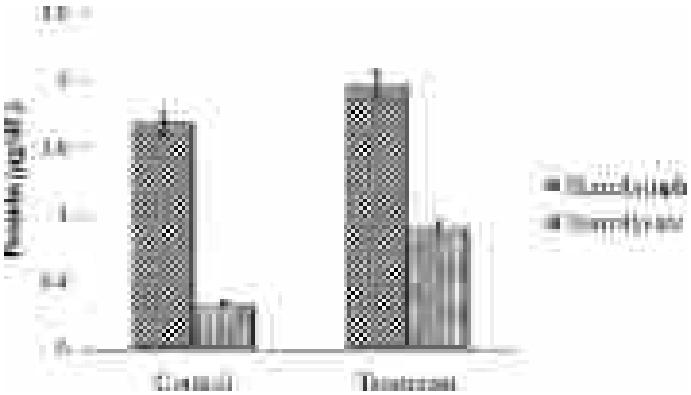
Table 5. Antibacterial activity of high molecular protein in freshwater zooplankton *Mesocyclops leuckarti* hemolymph against bacterial pathogens

Samples	Zone of Inhibition in diameter (mm)			
	Klebsiella pneumonia	*Escherichia coli*	*Shigella flexneri*	*Staphylococcus aureus*
Control	-	-	-	-
Test Sample (S1)	27mm	20mm	17mm	19mm
Test Sample (S2)	29mm	25mm	20mm	25mm
Amickacin (Standard)	27mm	25mm	28mm	30mm

Table 6. Antibacterial activity of low molecular protein in freshwater zooplankton *Mesocyclops leuckarti* hemolymph against bacterial pathogens

Samples	Zone of Inhibition in diameter (mm)			
	Klebsiella pneumonia	***Escherichia coli***	***Shigella flexneri***	***Staphylococcus aureus***
Control	-	-	-	-
S1	14mm	-	-	-
S2	18mm	15mm	12mm	13mm
Amickacin (Standard)	30mm	25mm	25mm	25mm

Fig 40. Protein Estimation of Freshwater Zooplankton Hemolymph and Hemocyte lysate of *Mesocylops leuckarti*

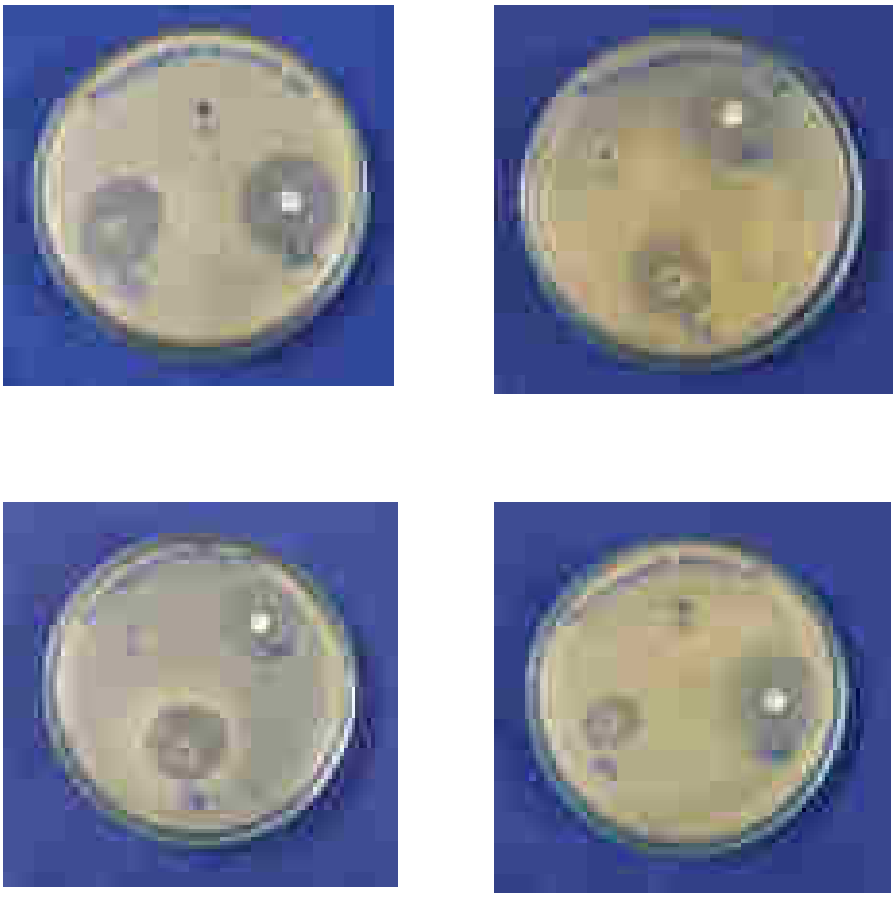


Control – Zooplankton culture without Cow urine distillate Treatment – Zooplankton culture with Cow urine distillate

Plate 22. Antibacterial activity of high molecular protein of *Mesocylops leuckarti* hemolymph cultured without cow urine distillate

S.A: *Staphylococcus aureus;* K.P: *Klebsiella pneumonia;* E.C: *Escherichia coli;* S.F: *Shegella flexnieri*; S1: Hemolymph of plankton cultured without CUD; CUD: Cow urine distillate

Plate 23. Antibacterial activity of high molecular protein of *Mesocylops leuckarti* hemolymph cultured with cow urine distillate



S.A: *Staphylococcus aureus;* K.P: *Klebsiella pneumonia;* E.C: *Escherichia coli;* S.F: *Shegella flexnieri*; S2: Hemolymph of plankton cultured with CUD; CUD: Cow urine distillate

Fig. 41. Antibacterial activity of high molecular weight of protein in hemolymph

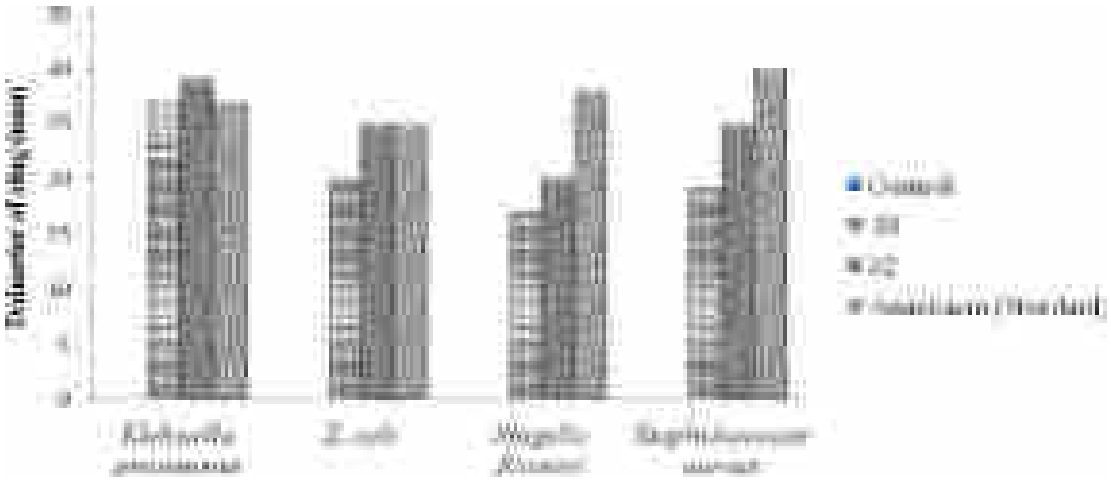
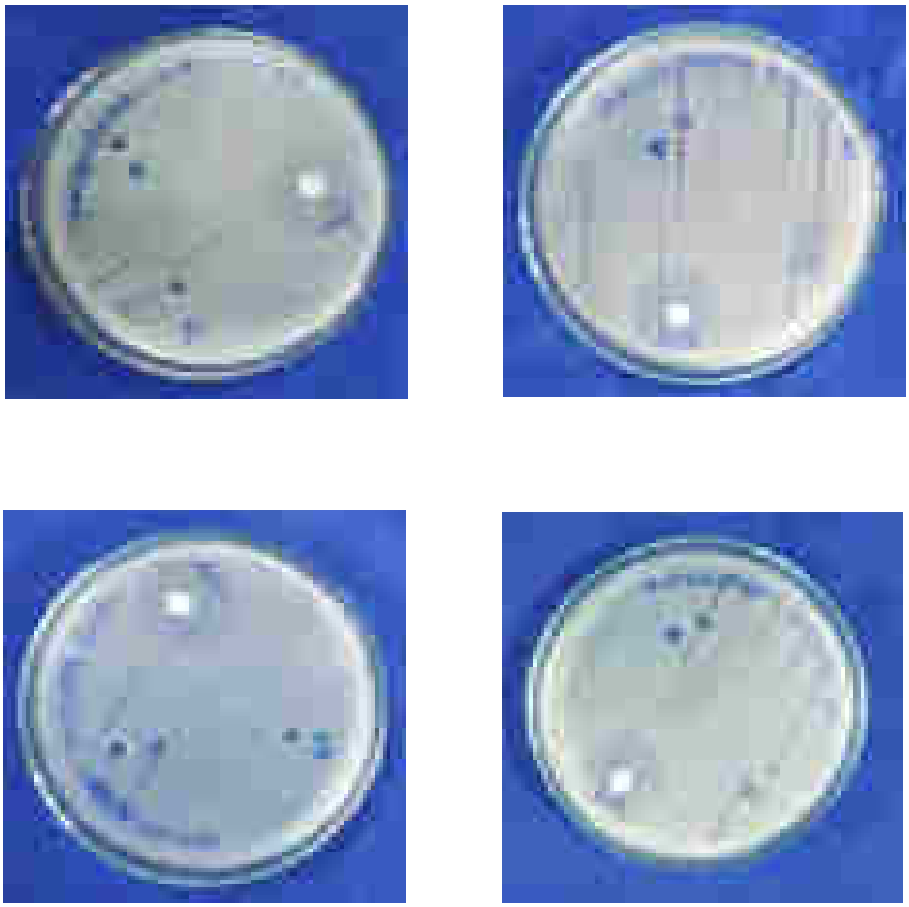
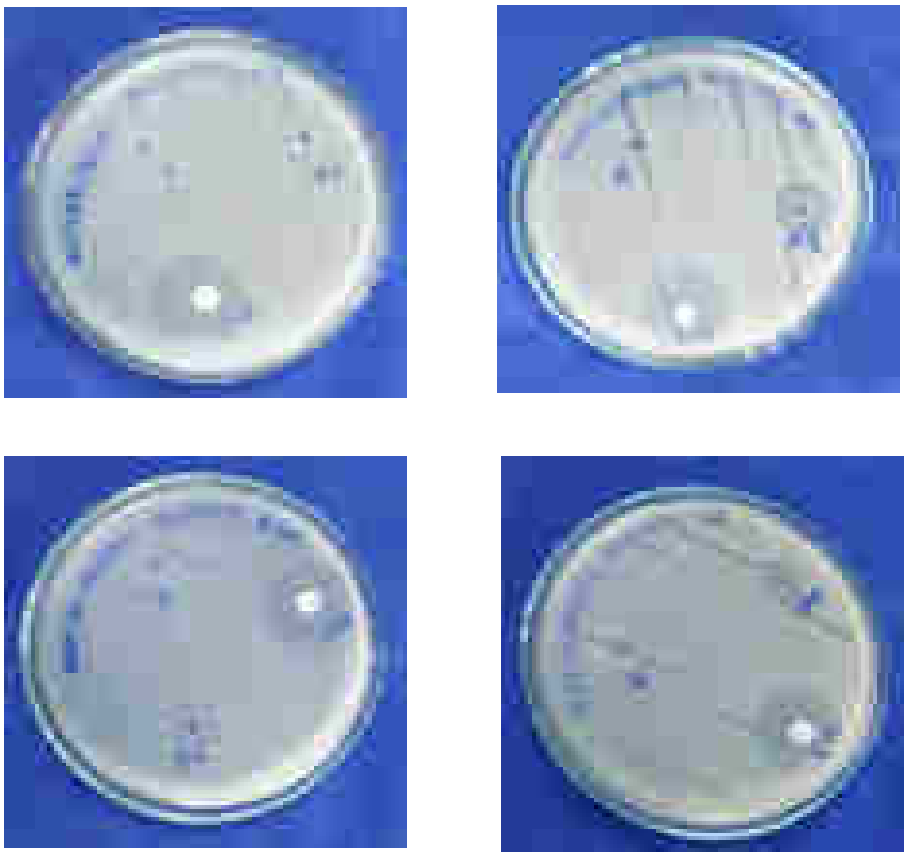


Plate 24. Antibacterial activity of low molecular protein of *Mesocylops leuckarti* hemolymph cultured without cow urine distillate



S.A: *Staphylococcus aureus;* K.P: *Klebsiella pneumonia;* E.C: *Escherichia coli;* S.F: *Shegella flexnieri*; S1: Hemolymph of plankton cultured without CUD; CUD: Cow urine distillate

Plate 25. Antibacterial activity of low molecular protein of *Mesocylops leuckarti* hemolymph cultured with cow urine distillate



S.A: *Staphylococcus aureus;* K.P: *Klebsiella pneumonia;* E.C: *Escherichia coli;* S.F: *Shegella flexnieri*; S2: Hemolymph of plankton cultured with CUD; CUD: Cow urine distillate

Fig. 42. Antibacterial activity of low molecular protein in hemolymph

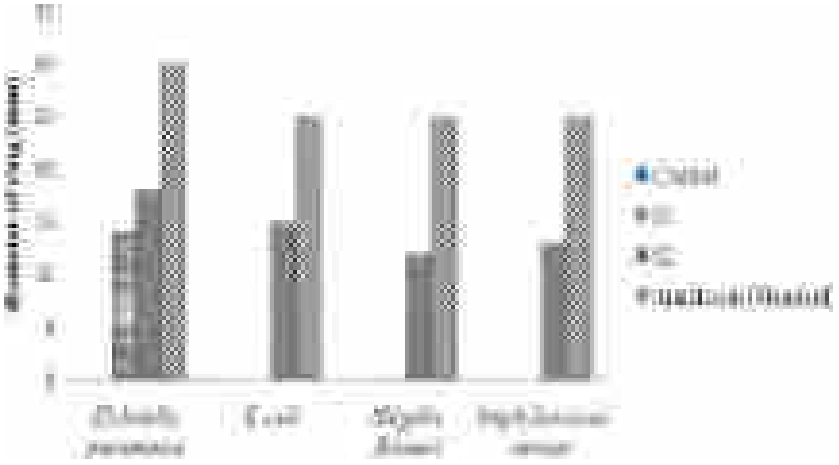
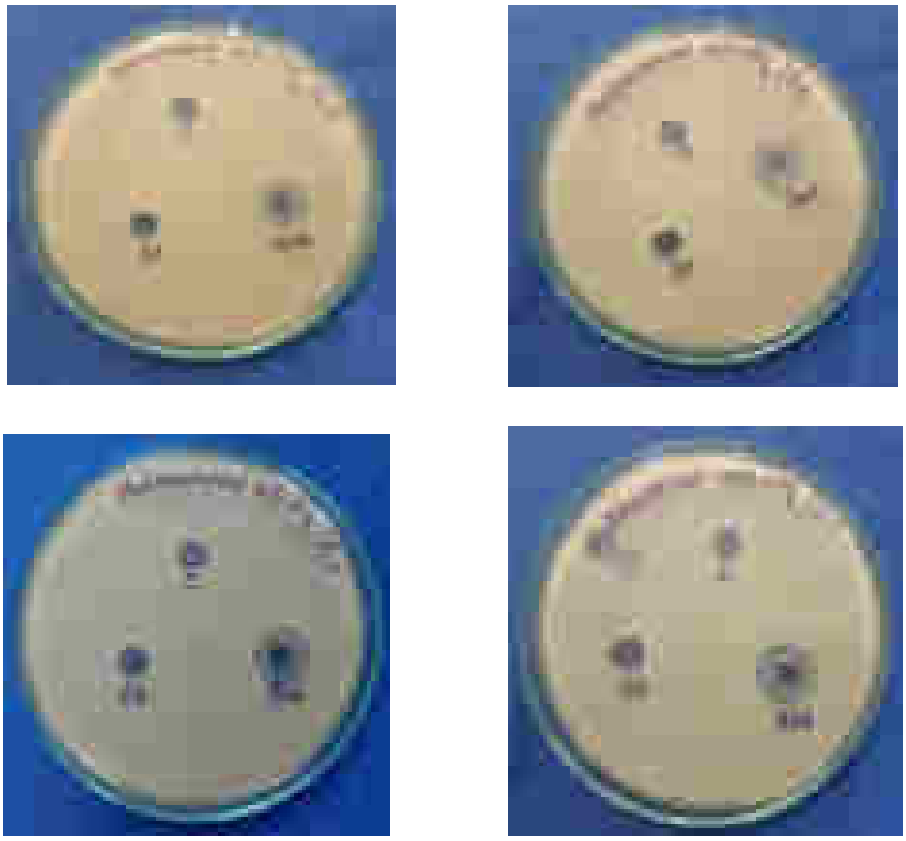
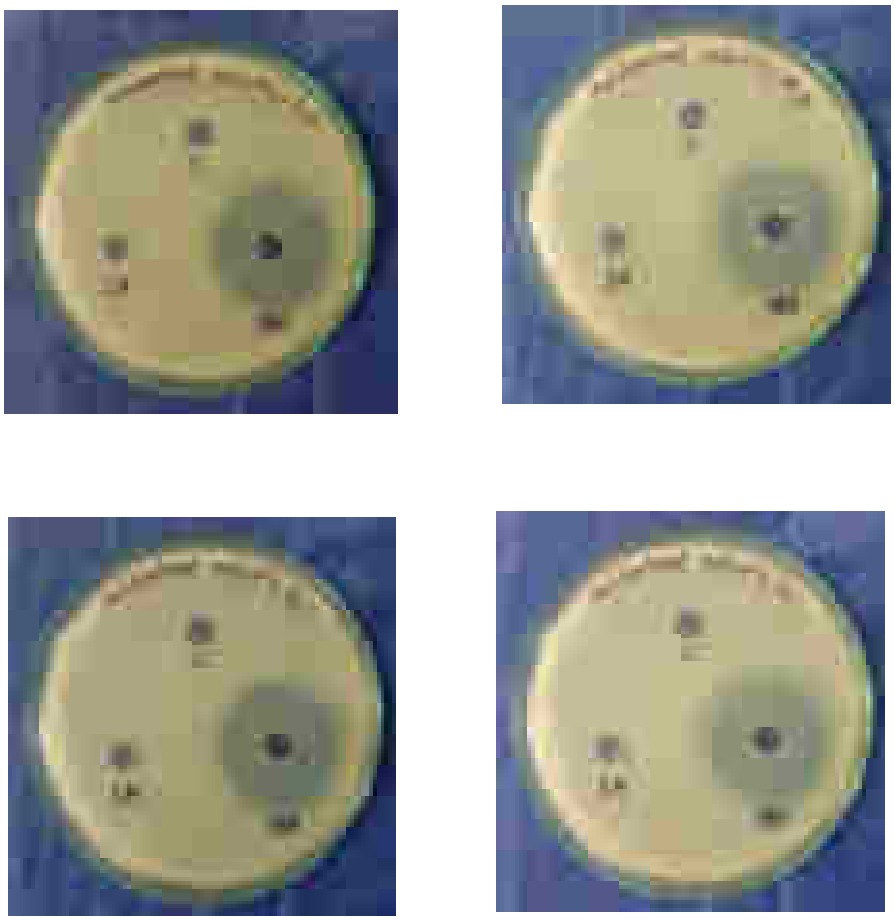


Plate 26. Antibacterial activity of high molecular protein of *Mesocylops leuckarti* hemocyte lysate cultured without cow urine distillate



S.A: *Staphylococcus aureus;* K.P: *Klebsiella pneumonia;* E.C: *Escherichia coli;* S.F: *Shegella flexnieri*; S3: Hemocyte lysate of plankton cultured without CUD; CUD: Cow urine distillate

Plate 27. Antibacterial activity of high molecular protein of *Mesocylops leuckarti* hemocyte lysate cultured with cow urine distillate

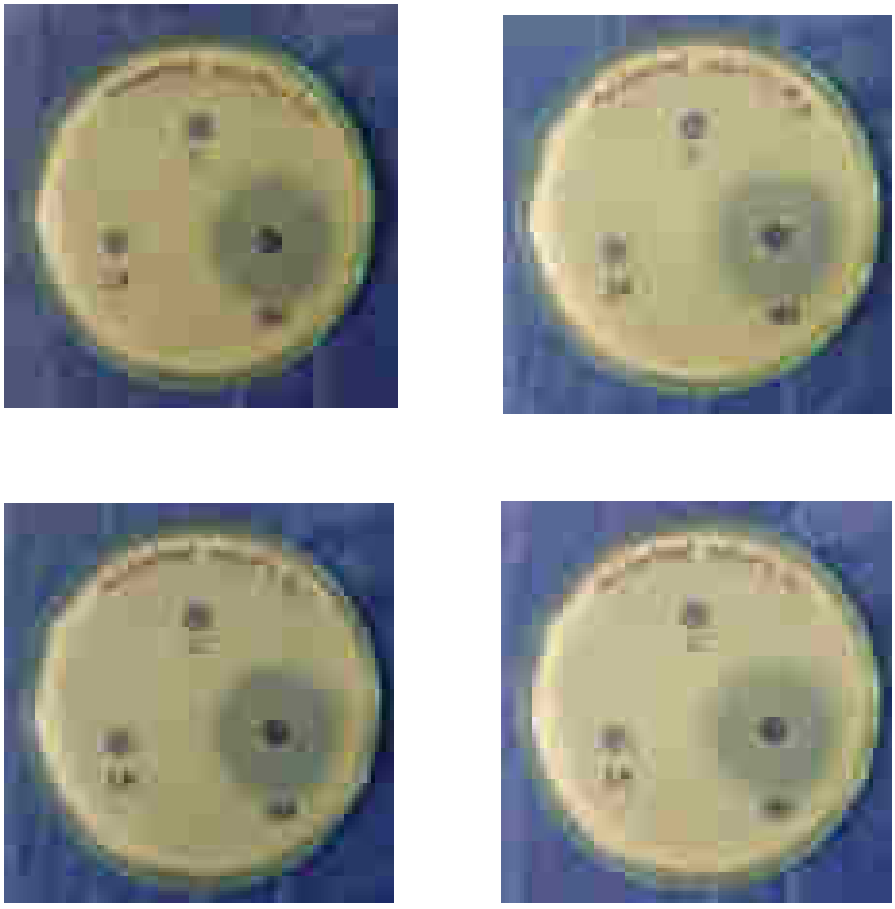


S.A: *Staphylococcus aureus;* K.P: *Klebsiella pneumonia;* E.C: *Escherichia coli;* S.F: *Shegella flexnieri*; S4: Hemocyte lysate of plankton cultured without CUD; CUD: Cow urine distillate

Plate 28. Antibacterial activity of low molecular protein of *Mesocylops leukarti* hemocyte lysate cultured without cow urine distillate

S.A: *Staphylococcus aureus;* K.P: *Klebsiella pneumonia;* E.C: *Escherichia coli;* S.F: *Shegella flexnieri*; S3: Hemocyte lysate of plankton cultured without CUD; CUD: Cow urine distillate

Plate 29. Antibacterial activity of low molecular protein of *Mesocylops leuckarti* hemocyte lysate cultured with cow urine distillate



S.A: *Staphylococcus aureus;* K.P: *Klebsiella pneumonia;* E.C: *Escherichia coli;* S.F: *Shegella flexnieri*; S4: Hemocyte lysate of plankton cultured with CUD; CUD: Cow urine distillate

The analysis of hemolymph and hemocyte lysate fractions by SDS-PAGE indicated the molecular weight of antimicrobial proteins were approximately in the range between 14kDa to 100 kDa (Plate 20). The appearance of single band in each lane in 12% gel indicated their purity.

Fig 40 depicts the total amount of protein isolated from the hemolymph and hemocyte lysate of the zooplankton *M. leukarti* reveals 68 and 96µg/dl of protein were isolated from the hemolymph of zooplankton cultured with cow urine distillate and without CUD respectively. Similarly, the amount of protein observed in the hemocyte lysate was found to be 0.9 µg/dl from the zooplankton cultured with CUD and 0.3 µg/dl from the culture free from CUD.

Antimicrobial activity of proteins obtained from hemolymph and hemocyte lysate of *M. leukarti* was estimated by agar well diffusion method. The results indicated different zone of inhibition against the selected human pathogens such as *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Shigella flexneri*. It is evident from the results (Plate. 21 – 28; Table. 5 & 6) that the microbial pathogens are more susceptible to hemolymph proteins in the presence of CUD and the hemocyte lysate derived proteins did not show any antibacterial activity either in the presence or in the absence of CUD. Table 1 presents the zone of inhibition exhibited by high molecular weight proteins of hemolymph. Inhibitory activity against gram positive and gram negative bacteria were evident from the results with most pronounced effect being observed with K. pneumoniae exhibiting a zone of inhibition of 27mm (Sample S1) and 29mm (Sample 2). Among the tested microbes the gram-positive bacteria *S. aureus* showed least susceptibility to hemolymph protein. Table 6 depicts the inhibitory activity of low molecular weight proteins of hemolymph and inhibitory activity was recorded once against *K. pneumoniae* (14mm) when the

plankton were cultured in the absence of CUD. But the antibacterial activity of the low molecular weight proteins of *Mesocylops leukarti* cultured in the presence of CUD was found to be enhanced and zone of inhibition against all the tested microbes were evident (Sample S4). Table 5 and 6 represents the antibacterial activity of high and low molecular weight proteins obtained from the hemocyte lysate of *M. leuckarti*. The proteins of hemocyte lysate are found to be ineffective against the selected microbial pathogens as no zone of inhibition was observed.

DISCUSSION

Zooplankton is ecologically and economically significant group of organisms. They have a centre place in the aquatic food chain and biogeochemical cycles. Also, they play a vital in fisheries as they form the first food for many larvae and fishes. Hence the care for their population studies have importance on both environmental and economic issues.

Physicochemical parameters of pond water

There is a growing interest in culturing this zooplankton in mass to find a solution for providing live feeds to fishery farmers in a large scale. For this purpose, the prime requirement is to find the environment conditions in which this zooplankton flourish. Hence a complete study of physicochemical parameters of the local water bodies in one complete seasonal cycle forms the first step in any planktonic studies. The study of Zooplankton diversity in this period gives us an idea of the dominant species of the locality and suggests the candidate for mass culture.

Hence in the present study the work was initiated with analysis of physic chemical parameters of three local water bodies (Ponds) in all four seasons from 2017 January to December 2017. The results revealed that the water quality parameters were significantly influenced by seasonal variations. The pearson correlation matrix showed correlation between various physico chemical parameters. Fig (6) exhibits that the water temperature is almost similar in all three study ponds with a similar pattern of seasonal variation. Fig (7) clearly conveys that the salinity of pond 3 (Sei pond) has least salinity and Pond 1 (Mathi pond) has highest salinity. The same pattern is seen in pH, (Fig 8) Nitrite (Fig 13) Nitrate (Fig 14) Ammonia (Fig 15) Total nitrogen (16) Inorganic phosphate (17), Total phosphate (18) and Silicates (Fig 19)

also. But, the dissolved oxygen content is least in the Mathi pond. So, the biological oxygen demand is higher. The level of Dissolved oxygen remained the same irrespective of the seasonal variations. There is an unusual rise in ammonia and total nitrogen content of mathi pond from June to November.

Among the three ponds studied, the Pidari pond and Sei pond showed a constant physicochemical parameter in water quality, while the Mathi pond showed variations in different seasons with much higher variations after June. The reason might be due to the fact that the city kumbakonam celebrated the Masi Maham festival in 15th February, 2016 to 7th March, 2016. It is a festival of bathing ceremony, that took place once in 12 years. More than 40 lakhs of people visited and took bath. The Government released substantial funds to dredge all the ponds in kumbakonam. After the cleaning process, the ponds were protected from anthropogenic interferences with barry guards. But the Mathi pond being a natural pond was left unprotected and was the source of water for many anthropogenic practices like bathing, washing clothes, cattle bathing etc. Hence the water quality was maintained in the same level in other two ponds while, the mathi pond after period of one year showed fluctuations that maybe due to anthropogenic interferences.

The water quality is constantly changed by natural biological processes like respiration, photosynthesis and decomposition. These processes are influenced by water temperature. There is a positive correlation between temperature and these biological processes. The results of these processes inturn influence the chemical composition and physical characterization of water.

According to Murugesan *et al.,* (2004) the increasing water temperature was responsible for the increase in chemical and biological action in water bodies and reduction in solubility of gases. In the present investigation, a positive correlation is

observed between temperature and salinity, pH, TSS, NO_3, NH_4, IP, TP and Silicates. Similarly, a negative correlation is observed between temperature and DO. Hence during high temperature a strong chemical oxygen demand has been created.

Zooplankton diversity

Kumbakonam is known for its nick name "Pond city" due to the presence of large number of ponds mainly because of the presence of lot of temples. These water bodies are mostly manmade ponds constructed for a temple as temple pond. There are also some natural ponds. Some of these ponds are degraded historically by human action or due to succession. Restoration of these ponds happened recently in 2016 at the event of Mahamaham. Zooplankton can be a proof for assessing the success of restoration (Anton-Pardo *et al.,* 2013) since their changes in abundance or diversity can be considered as a response to variations in water quality and can reflect modification produced in lower and higher trophic levels.

The data obtained from the study ponds recorded 27 zooplankton species as the maximum in Mathi pond which is a natural pond. The other two ponds are restored ones, of which Sei pond has 17 species and Pidari pond has 14 Species. In all the ponds 3 species were found to be common inhabitants. They are four Copepods *Sinodiaptomus sarsi, Thermocyclops hyalinus, Cyclops vernalis, Eucyclops speratus;* three Cladocerans: *Diaphanosoma sarsi, Daphnia magna* and *Moina micrura;* Three Rotifers: *Brachionus calciflorus, Brachionus rotundiformis* and *Brachionus cadus pesonatus* and three Ostracodans: *Cypris protubera, Eucypris bispinosa* and *Cyprinotus nudus.*

The diversity indices projected Mathi pond as the well diversed and species rich, abundant pond among the three ponds studied. The reason might be that it is a

natural permanent pond were the other two are restored ponds. They may reach the maximum diversity in future. Even Mathi pond shows the maximum zooplankton population density which is nearly double that of Pidari pond and 1.7 times higher than that of Sei pond. In general copepods are found in large numbers followed by Rotifers and then Cladocerans, Ostracodans are the minority groups in all the three ponds. In all the three ponds the pattern of seasonal influence on zooplankton population density is found to be similar with lower density during summer and higher during winter seasons.

The number of copepods is found to decrease during hot summer compared to other seasons. But Ostracodans number increased to some extent comparatively in summer. The Rotifers and Cladocerans do not show much variations in these density between seasons. But these results are deviating from those present in literature. Perumal *et al.,* (2009) found that maximum zooplankton density was found during summer. The variation recorded in estuary in contrast to the pond in the present study.

A study in tidal backwater of east coast in India by Sahu *et al.,* (2013) also showed high zooplankton biomass during premonsoon season (i.e) Summer. Another study on the impact of seasonal changes in zooplankton biodiversity of Ukkadam lake, Coimbatore by Manickam *et al.,* (2018) showed that the density was high in summer. A similar result was shown by Beshini *et al.,* (2017) in a temple pond at Thiruvotriyur, Chennai. Krishna and Kumar, (2017) showed that higher population density of the zooplankton was observed during both monsoon and low population during density was observed during summer and post monsoon in selected ponds of Andhra Pradesh. This result is in line with our data. The authors attributed the abundance of zooplankton in the monsoon with the abundant food sources from the

run off. MuyLaert *et al.,* (2003) also observed that the zooplankton abundance frequently reached their peak during the wet season in ponds.

Ikpi *et al.,* (2013) correlated low predation rate by fishes during wet season with the high zooplankton density. In the present study the peak values of rainfall were recorded in the month of November 2017 and lowest was recorded in the month of Februaury, 2017. Sarkar *et al.,* (2020) reported the increase in number of zooplankton during the early rainy season and then started decreasing as the rainy season postseason in west Bengal. The water temperature ranged from 26^{o}C to 34^{o}C during the present study period. Temperature is one of the important environmental factors that influences fauna and flora (Murugesan *et al.,* 2004). Meerhoff *et al.,* (2012) discussed that shallow water bodies are very similar to climate change and their influence the community traits. Yvon-Duvocher *et al.,* (2010) found that temperature affects a myriad of biological processes including individual growth and respiration water. It was also studied and found that warming contribute to distribution of species, affecting diversity and biomass (Woodwad *et al.*, 2010a) decline in cladoceran and copepod species richness was reported with increasing water temperature (Shurin *et al.,* 2010).

Gyllsthrom *et al.,* (2005) showed higher zooplankton biomass in colder region of the globe and lower levels in warmers lakes. Hence according to the recent studies, our data of the present investigation that zooplankton density is higher during post monsoon and winter. Lower density was observed during summer season. The water temperature in post monsoon season might be the optimum for the zooplankton of this region and also there might be less predation. The availability of more food resources, less pollution like factors may also have played upon.

To conclude, the zooplankton diversity studies showed that copepods and rotifers are the common inhabitants of the three study ponds. Rotifers have been identified as indicators of water pollution (Arora, 1966). Hence it is time to take care of the remedial measures. The *Mesocyclops leuckarti* was found to be the dominant species in two ponds. Though not dominant, *Sinodiaptomus sarsi* is found in good numbers in all the three ponds of investigation. Hence as the next phase these two organisms were isolated separately to try for pure culture and mass culture and for molecular identification studies.

Molecular identification

According to Blanco-Bercial *et.al,* (2014). The identification of zooplankton is challenging even for expert taxonomists. Hence the barcode region of the mitochondrial cytochrome c oxidase subunit (COI) gene is found to be useful and unequalled diagnostic character for species level identification of copepods Su Youn Baek *et al.,* (2016) stated that "molecular approaches to species identification have allowed rapid detection, discrimination and identification of cryptic or sibling species based on DNA sequence data". Hence after morphological identification using traditional methods with the aid of manuals the two dominant species were isolated and cultured separately for DNA sequencing. After obtaining the nuclear sequence, the molecular identification was carried out using mitochondrial COI gene as marker. Laakmann *et al.,* (2013) compared different molecular identification methods of copepods on the basic of nucleotides and proteome fingerprints. As per their findings and conclusion, "DNA investigation allow an ambiguous identification with sequences representing valued entities irrespective of the life history/developmental stage, origin and parts of organism. Once DNA compared with 18S r DNA". Based on

their findings, in the present study the sequence of COI gene has been used as the markers for molecular identification.

The problem in identifying *M. leuckarti* and *T. oithonoides* is not straightforward, especially in Continental European lakes, where 3 – 6 cyclopoid copepods often coexist in the pelagial, and in which population share dominated by larval and juvenile instars during much of the year. Analyses of life cycles is crucial in ecological research, and considering the 12 different developmental stages, the work often seems over whelming or practically insurmountable.

Nilssen and Waervagen, 1982 poidae indet, or Cyclops spp the latter ctegoryusually including several species both of cyclops, *Mesocyclops* and *Thermocyclops.* This is ecologically unfruitful, since in nearly 100% of the cases, cyclopoid copepods are present in the pelagial of most lakes all over the world.

More serious problems arise when the autecological details of the different species are concerned and when the species are falsely identified. Figure 2 presents the most important "field" (stereomicroscopic) species characters of *Mesocyclops lecukarti* and *Thermocyclops oithonoides.* Notably are the shape of furcal setae, their internal length relationships, the position and shape of the egg sacs of the gravid females, the general body shape, and the size of the animals. In addition, some ecological parameters are important, notably the altitude of the recorded site, the number of eggs per egg sac, and the habitat distribution of adult females and diapausing copepodids. *Mesocyclops leuckarti* is considerably larger, by approximately 20 -40% (total body length: 1.0 – 1.3 vs 0.7 – 1.0 for *hermocyclops oithonoides*).

The phylogenetic tree constructed with (Fig 29, 31) and Blastn results confirmed the molecular identification of the species. However, the 98% similarity between 18S rRNA genes of *Mesocyclops leuckarti* and *Mesocyclops pehpeinsis* needs to be resolved in future. Similar such issue was reported by Youn Back *et al.,* (2016). In their studies a clearcut resolution of species identification was not provided by COI marker in four cases of *Mesocyclops dissimilis, Mesocyclops pehpeiensis* and *Oithona davisae, Oithona similis* in cyclopoida, *Oithona japonica, Pseudomyicola spinosus* in Poeciostomatoida and *H. japonica, c. quadrates* in Siphonostomatoida. The researchers also expressed that "as 18S rRNA data of copepods increase, the development of universal primers specific to copepods might be possible".

Survivability of different copepod species in culture

The experiments conducted with the two dominant species identified as dominant populations in the local study ponds revealed that *Mesocyclops* is more sustainable under lab conditions. *Sinodiaptomus sarsi* is observed in appreciable numbers in all the three study ponds. However, the species was unable to survive in culture under lab conditions. But *Mesocyclops leuckarti* though observed only in two ponds was able to survive and sustain in culture under lab conditions. Throughout the experimental period. Hence it has been selected as the candidate species for further experimental studies with cow urine distillate and other immunological studies.

Experimental studies with Cow Urine Distillate on *Mesocyclops leuckarti*

Mariyappa *et al.,* (2018) recorded that the use of cow urine with cow dung as pond fertilizer not only increased good growth of plankton but was also reflected on fish production.

FAO (2020) has pointed out that “live stock are a key resource for economic growth in many countries both at national and household level. Animals are important aspects and an essential source of income for livestocks- keeping households. Moreover, live stock can also supply inputs for crop production such as fertilizer or animal fraction”. The integrated fish cum cattle farming is an old age system of practice. It cuts down the cost of fertilizer and feed in aquaculture system (Asif chankrabathi, 2014). The TamilNadu Agriculture University. TNAU Agritech portal also advices the utility of cattle feces and urine for integrated fish farm practices (agrtech.tnau.ac.in/fishery).

Matt Kaplan (2007) has reported even human urine can be used for nourishing planktons. Ali (2006) showed that cattle urine has significantly influenced the growth of the rotifers *Brachionus calyciflorus* in culture. Praveena *et al.,* 2020 recorded that Gir cow urine distillate has increased the population density of cyclopoid copepods in culture and also enriched the biochemical composition of these organisms.

Hence based on the above scientific studies, cow urine distillate has been experimented as a growth promoter for plankton culture. The two zooplankton species that were identified as common and dominant in the study area are *Mesocyclops leuckarti* and *Sinodiaptomus sarsi*. Of these two *Sinodiaptomus* could not survive in laboratory conditions. Hence *Mesocyclops* after conducting survival experiment, was selected as the experimental organisms for further studies. Several recent studies strongly recorded the supremacy of Indian cow breeds over cross bred cows in their medicinal properties of milk (Rajeswari *et al.,* 2016; Kumar *et al.,* 2018), cow urine and cow dung (Rajeswari *et al.,* 2016). Hence Gir cow which is one of the unique Indian breeds has been selected for the source of cow urine distillate in the present study.

Since Sattanathan and Venkatalakshmi (2016) showed 5% of cow urine as toxic to *Labeo rohita* fish is inspite of their good growth promoting and immuno stimulatory effects at lower doses; the need for finding the toxicity level of CUD on plankton was understood. Accordingly, 1% CUD was found to cause 100% mortality and 0.6% causes 50% mortality on *Mesocyclops leuckarti*. Since the micro crustaceans are less complex and small organisms, the CUD caused mortality at 1% itself while it is toxic to fishes at 5% only. Newman *et al.,* (1994) quantified the size – dependent toxicants impact. Wright and Welbourn (2002) has documented that toxicity of a toxicant is influenced by morphological and biochemical differences between or among organisms of different size and taxonomic groups.

The toxicity of CUD in *Labeo rohita* at 5% concentration (Sattanathan and Venkatalakshmi, 2016; Priya and Venkatalakshmi, 2016) and at 1% in *Cihinus mirgala* (Padmapriya and Venkatalakshmi, 2015) is supportive evidence as CUD caused 100% mortality in 1% concentration in copepod *Mesocyclops leuckarti*. Though it has shown toxic effects at higher concentrations, its beneficial effects have been revealed at lower concentrations in aquaculture (Durga and Venkatalakshmi, 2015). Hence CUD was experimented at lower concentrations for its effect on the growth and reproduction in terms of survival, population density and nauplii production rate.

Effect of CUD on Survival

The optimal concentration of CUD for plankton culture practices was assessed with a trial experiment consisting of four different concentrations lesser than the toxic level – 0.2%, -.1%, 0.05% and 0.025%. A dose dependent effect was exhibited by CUD on the survival of *Mesocyclops leuckarti* with the maximum survival at 0.025% concentration and least survival in at 0.2% concentration. CUD has experimentally

shown to have good growth promoting activities in plants (Savita *et al.,* 2015) broiler (Rakesh *et al.,* 2013; Tadavi *et al.,* 2017) and fish (Priya and Venkatalakshmi, 2016, Padmapriya and Venkatalakshmi, 2014, Sattanathan and Venkatalakshmi, 2015) at 0.1% concentration. However, the same 0.1% concentration is not good in the case of copepods. The maximum effect was elicited by a still lower concentration (i.e) 0.025%. These results indicate that CUD might have a size dependent activity. However, it needs further research before it is confirmed.

Effect of CUD on Nauplii Production Rate

A dose dependent effect was evoked by CUD on the nauplii production rate of *Mesocyclops leuckarti*. There was an inverse dose dependent effect recorded. (i.e) as the dose decreases, the biological effect increases. The least concentration of 0.025% elicited the maximum response with 28 nauplii production per female. Nauplius is the earliest and most basic type of a crustacean larva. Copepod nauplii are the major prey of the early stage of fish larva and can influence fish population dynamics (Garrido *et al.,* 2015) and constitute a key link between the microbial loop and higher trophic levels. (Roff *et al.,* 1995). Hence the nauplii production rate is considered as an important factor for a live feed. It reflects the viable egg production ability of the copepod. Hence the ability of CUD to motivate the naupli production rate suggests CUD as a possible growth promoter in aquaculture practices.

Effect of CUD on Population density

A similar inverse dose dependent effect of CUD could be observed on the population density also with T1 showing the maximum results and T4 exhibiting minimum effect. Kimmerer *et al.,* 2014b pointed out that primary productivity is positively related to growth and reproductive rates of zooplankton. Their experiments

proved that low salinity affects the growth rate of copepods. Similarly the bio mass of *Acartia* species was shown to be influenced by increase of water temperature (Mollmon *et al.,* 2000) Musialik-koszarowsha *et al.,* (2019) analysed and confirmed that the environmental factors like temperature and salinity influences the population dynamics of zooplankton. Hence the addition of CUD might have increased the salinity due to its constituents and thus increased the population density of *Mesocyclops leuckarti*. This could be further analysed in laboratory trials.

Effect of CUD on Biochemical composition

The growth of aquaculture industry depends on proper farm management which includes the quality of feed. Feed should supply all the requirements of the organism for the maintenance of physiological function. The proximate analysis can measure nutrient contents of the feed. Proximate analysis is the quantitative analysis of different macronutrients in feed. It was developed in 1860 by Hennberg and Shohman in Germany. Zooplankton copepods are utilized in particulars in fish larviculture as live feeds mainly because of their nutritional value. Investigations on copepod culture methods and their use as natural food for fish larvae might help to develops more accurate techniques for large scale culture of copepods, thus making feasible their use as a high quality and early digestible food (Shettu and Yasmin, 2009). Natural zooplankton constitutes ideal food for fish larvae because of the presence of vital enzymes that help in the function of the digestive tract (Ronnestad *et al.,* 1999) Nilssen and Waervagen (2000) showed that *Mesocyclops leuckarti* is more suitable for fish predator than other copepods. Priyanka *et al*., (2017) analysed biochemical composition of freshwater zooplankton *Mesocyclops leuckart.* It was shown that *Mesocyclops leuckarti* contain minimum of glycogen content, maximum of protein and lipid when compared with *Brachionus calyciflorus* and *Moina micrura.*

Riccanrdi and Margoni (1999) summarized the literature data on the range of variation of zooplankton biochemical composition. According to that fresh water copepods contain 2.9 to 5.1% ash; 43-64% carbon, 6.2-15.1% nitrogen; 6.9 – 7.2% hydrogen; 1.0 – 41.2% Carbohydrates; 6.0 – 43.2 % Lipids and 17.6 – 76% proteins. The result of the present study dwells well with in this range. In the trial experiment to assess the efficiency of CUD in influencing the nutrient value of the zooplankton, a successful result was obtained at 0.025% concentration CUD treated groups with highest protein, carbohydrate, lipid contents. Hence by using CUD as a water additive, the nutrient value of the line feed could be enhanced.

Immunological studies in *Mesocyclops leuckarti*

Evolution of life has happened from single cell organisms to multicellular complex organisms. In this process, coevolution is likely to happen when different species have close ecological interaction with one another. Lake 2009, discussed that all complex organisms are the result of coevolution between two or more other species. The coexistence of two different species results in different animal relationships like commensalism, mutualism and parasitism. Among this, the host-pathogen relationship develops a compulsion for evolving adaptation to protect by the host and to attack by the pathogen. Immune system is one such adaptation for the host organisms to protect themselves from the pathogens. The weapons to fight against invading pathogens are found in the simplest eukaryotes and more complex animals. And then when multi cellular creatures evolved, they were able to devote specialized cells to tasks such as engulfing bacteria and it uses (John Trovis, 2009).

In due course, crustaceans too have evolved a simple defense mechanism mainly comprised of innate immunity (Lorena vequez *et al.,* 2009). Smith and Chisholm (1992 and 2001) stated that the circulating hemocytes play important roles

by direct sequestration and killing of infecting agents and synthesis of bioactive protein molecules.

Copepods, a micro crustacean groups and hosts of several species of parasites; the most important being the microspotidians, nematodes and cestodes. But surprisingly little is known about infection processes and potential anti-parasite defence of copepods (JoachinKurtz., 2007) copepods are the intermediate hosts for *Dracunculus medinesis*, a human parasite; *Anguillicola crassus* and camallanus sp., the fish-parasitic nemetodes (Levesen and Jokabcen, 2002) and also they act as 'transport hosts' from nemetodes to nematodes (Koie, 2001) Joachin Kurtz., 2007 experimentally showed that there is specificity and memory in the immune mechanism of copepod *Macrocyphins albidus* against nematode parasites in terms of reduction in re-infection for sibling parasites compared to prior exposure to unrelated species. Little *et al.,* 2003 also showed that there might be maternal transfer of specific protection. It was suggested that though copepods slowly rely on innate immunity, there should be alternative mechanism that might establish specific recognition within innate immune system. Franz and Kurfz (2002) suggested that tapeworms directly manipulate copepod behavior. Hoffmann and Reichhart (2002) studied the *Drosophila* innate immunity. Pasternate *et al.,* (1995) explained the changes in metabolism and behavior of the fresh water copepod Cyclops, *Strenuus alyssouram* infected with Diphyllobothrium spp. However, the knowledge of the immune system in these micro crustaceans is rare. Change – Bum Jeong *et al.,* (2015) showed the increase in transcriptional levels of dorsal and dorsal like genes upon exposure to immune modulators in the cyclopoid copepod *Paracyclopina niana*. Among micro crustaceans a handful of studies are available for the hemocyte mediated immune mechanisms in Daphnia (Shan Liu, 2020). However, there is no

reports available for copepods regarding hemocytes (Shan Liu, 2020). Hence the observation of hemocytes in the freshwater copepod *Mesocyclops leuckarti* is the first of its kind to the extent of our knowledge.

The circulating hemocytes of crustaceans consists of three main types each with their own role in immunity and homeostasis (Rowley, 2016) Praveena and Venkatalakshmi (2019) have reported isolation and identification of hemocytes in the cyclopoid copepod. A premilinary method of isolation of hemolymph and hemocytes have been reported. The types of hemocytes identified were hyaline cells, semi granulocytes and granulocytes that matches with the types of hemocytes in other crustaceans.

Rowley (2016) suggested that phagocytosis is nodule formation and the key mechanisms for the clearance and elimination of microbes and unicellular parasites by hyaline cells and semigranule cells. Similarly, it was reported that crustaceans have a humoral immune system comprised of prophanol oxidase system (Cerenius *et al.,* 2010 a, b; Charoensapsri *et al.,* 2011; Sivakamavalli and Vaseeharanm 2013); antimicrobial peptides and proteins (Smith, V. J *et al.,* 2010; Hou *et al.,* 2014; Liu *et al.,*., 2015); and lectins (Sivahamavalli and Vaseeharan, 2013). Hence based on these reports, the attempt to isolate antimicrobial peptides from *Mesocyclops leuckarti* was made. The protein fractions of high and low molecular weight from hemolymph and hemocyte lysate were separated and tested for their antimicrobial activity (Praveena *et al.,* 2020). The high molecular weight protein fraction obtained from hemolymph showed effective antimicrobial activity, while the low molecular fraction showed antimicrobial activity against *Klebsialla* alone and no effect was pronounced on other pathogens. In contrast, the lysate of the hemocytes had no effect on the pathogens experimented. This shows that the defense mechanism of copepods is extracellular

and is not intracellular. The peptides derived from the hemolymph of invertebrates exhibit a broad spectrum of activities (Hajirasouli and Pazooki, 2014) and these peptides mainly target the bacterial membrane. It is confirmed in the present study and is documented the antimicrobial property of the hemolymph proteins from freshwater copepod. It was found that the hemolymph proteins are more potential against gram negative bacteria than gram positive bacteria. Among the pathogens tested, *Klebsiella pneumonia* more susceptible. The antibacterial proteins isolated from other crustaceans were recorded for their broad-spectrum activity against both gram positive and gram-negative species of bacteria (Destoumieux *et al.,* 1997, Bristsin *et al.,* 2015; Bachere *et al.,* 2000; Chang *et al.,* 2013). This shows the possible variations in the structure and property of the antibacterial proteins of copepods which needs further studies at molecular level. The hemolymph was observed to contain different proteins of molecular weight 53KDa and 19 KDa. But literature revealed 1.6 to 5.6KDa proteins in lobster *Homarus arrericannes* (Bristin *et al.,* 2015) Fredrick and Ravichandran, 2012 reviewed that hemocyte proteins of crustaceans ranged from 3.7KDa in *Calinectes sapidus* and 4000 KDa in *Scylla serata*. It was stated that large antimicrobial proteins of more than 100 amino acids are often lytic, nutrient binding proteins or specifically target microbial macro molecules. Small antimicrobial peptides act by disrupting the structure or function of microbial cell membrane. In the present study, it was found that the high molecular weight protein has more pronounced activity than the low molecular weight protein.

Immunostimulants are naturaly occurring compounds that modulate the immune system by increasing the host's resistance to disease (Bricknell and Dalmo, 2005) Apines – Amar and Amar, 2015 reviewed the use of immune stimulants in

shrimp culture. It includes synthetic, micro algal products plant derived products, nutrients and microbial products. Karunasagar *et al.,* (2014) reviewed the application of various immunostimulants studied in crustaceans.

Kallaya *et al.,* (2005) found that yeast extract acted as an immune stimulant and increased the hemocyte count in shrimp. Similarly, Takashi Imai and Yukinori Tahahash (2020) studied the effect of Kelp B glucan as the chemotaxis, phenol oxidase activity, super oxide production and resistance against white spot syndrome in *Marsupenaeus japonicas* hemocytes and found it was effective.

In the present study, cow urine distillate was used as the immunostimulant and it was proved to be prospective one from the results that the hemolymph of the CUD treated copepods have proteins with more antimicrobial activity. In the case of low molecular weight protein of the hemolymph, the CUD treated samples have antibacterial activity against both gram positive and negative bacteria while the untreated sample is active against *Klebsiella* alone. This shows that CUD has activated the antibacterial proteins of the hemolymph. Sahu Rekha *et al.,* 2017 has reviewed the benefits of cow urine. It has been patented for its effect as bioenhancer along with antibiotics for the control of bacterial infection (Dharma, 2005). Cow urine is a non toxic animal excreta with 95% water, 2.5% urea and 2.5% mixture of minerals, salts, hormones and enzymes (Bhadauria, 2002). It augments the immune system including B and T lymphocyte blastogenesis and Ig G, Ig A and Ig M antibody titers in mice. It also increases the secretion of IL-1 and IL-2 (Chauhan, 2004). CUD act as a good bio enhancer. A bio enhancer is an agent capable of enhancing the bio availability and efficacy of a drug with which it is co-administered, without any pharmacological activity of its own at the therapeutic dose used (radhawa *et al.,* 2011) Randhawa, 2011 pointed out that cow urine has bioenhancing activity for Rifampicin,

Zinc, Zn^{2+} (Khan and Srivastva, 2005) Kekuda *et al.,* (2010) documented that cow urine distillate is more effective as bioenhancer than cow urine. The results of the present study are also supportive for this. The high antimicrobial activity expressed in the peptides of heamolymph of *Mesocyclops leuckarti* treated with cow urine distillate proves the above said fact. It is also confirmed by the lesser or absence of antimicrobial activity in the cow urine distillate untreated samples.

It was widely accepted that *Mesocyclops leuckarti* has a remarkable cosmopolitan distribution (Sars, 1918 Kiefer, 1981; Dussart and Fernando, 1996; Dahms and Fernando, 1993).

Nilssen and Waervagen, 2000 studied deeply on *Mesocyclops leuckarti* and reported that they are more suitable for fish predation especially when carrying egg sacs.

Mesocyclops leuckarti has been observed to inhabit both acidic and saline habitants (Roen, 1957) Papinsha published the most detailed study on *Mesocyclops leuckarti* and stated that to occurred most of the year. Its life cycles showed two generation in a year following three life history patterns; one with a diapause lasting for 9 months (June to March); a second with diapause and third with diapause lasting for 5 months (October to March).

Papirniska (1985) studied the food preferences of *Mesocyclops leuckart* and showed that they can just survive on using detritus from bottom sediments and can feed on dead organisms.

Miiller (1785) stated that among the cyclopoids, the most important genera are Mesocyclops. They found in both temperate and tropical zoo geographical regions.

Hansen and Santer (1995) found that naupliar development time of *Mesocyclops leuckarti* was inversely related to attrached food concentration.

Herzig, 1983 indicated that *Mesocyclops leuckarti* developed rapidly at temperature above 18^{o}C.

Hence it is concluded that *Mesocyclops* could be considered as a suitable candidate for mass culture and development of technology for live feed production in large scale. In addition, their culture efficiencies and nutrient qualities could be improved by using CUD as a water supplement or additive various species of *Mesocyclops* are known to prey on mosquito larvae (Vdayava *et al.,* 2019). They are also dominant and suitable species in the local water bodies that can be cultured throught the year in all seasonal variations.

SUMMARY AND CONCLUSION

- The present study indicated that the rainfall has made remarkable changes on physico-chemical characteristics of the Freshwater local ponds studied – Mathi pond, Pidari pond and Sei pond.
- It is understood that the temperature and salinity significantly enhanced the zooplankton density and species diversity in freshwater local ponds.
- A total of 27 species were recorded in the three stations. All the species were recorded almost in all the seasons in appreciable numbers.
- Of these, 8 species of Copepods, 6 Rotifers, 6 Cladoceran and 7 ostrocods were recorded.
- Out of the three ponds studied, the Mathi pond showed high species diversity, abundance and zooplankton density while Sei pond shows the least.
- The dendrogram from cluster analysis showed that the stations Mathi, Pidari and Sei ponds were grouped at the highest and lowest level of similarities in four seasons.
- The molecular identification was done for 2 dominant copepods and the nucleic acid sequences were submitted to NCBI and the accession numbers are assigned as *Mesocyclops leukarti* (MN886516), *Sinodiaptomus sarsi* (MN886517).

- The selected freshwater copepod *Mesocyclops leukarti* has been experimented under different concentration of cow urine distillate to optimize the culture conditions.
- The cow urine distillate has toxicity over the copepods at higher concentrations and the LC_{50} value was found to be 0.6%.
- However, at 0.025% conc, of CUD has beneficial effects on the copepods in terms of population density, survival, biochemical composition and immunity.
- Three types of hemocytes were observed in the hemolymph of the copepods and they were identified as Hyalinocytes, Granulocytes and Agranulocytes.
- The copepods were found to have antibacterial activity in their hemolymph with high total protein content.
- Two kinds of protein were identified in the hemolymph and hemocyte lysate – The high molecular weight protein (100kDa) and low molecular weight protein (14kDa).
- The CUD has enhanced the quantity of the antibacterial proteins in hemolymph and hemocyte lysate.
- The high molecular weight protein of hemolymph and hemocyte lysate have antibacterial activity while the low molecular weight protein has either less or no effect.

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First Record of Isolation of Hemolymph and Identification of Hemocytes in a Cyclopoid Copepod

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Abstract

Based on the literature review done it has been noted that it is the first report of observation and identification of hemocytes from cyclopoid copepod. The hemolymph sample was taken by a preliminary standard method and hemocytes were isolated and identified under light microscopy using eosin stain. The hemocytes observed were clarified based on the presence or absence and relative size of cytoplasmic granules. Three types of hemocytes were observed and identified as granulocytes, semi-granulocytes and hyalinocytes. The differential counts of hemocytes were carried out in a Neubauer counting chamber. The results were recorded as 5-10% small granular hemocytes.

Keywords: *Crustaceans, cyclopoid-copepod, granulocytes, halinocytes, light microscope*

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INTRODUCTION

Copepods, microscopic crustaceans that inhabit lakes, ponds, rivers and ditches, serve as the main diet for many fish. Their significance as live feed in aquaculture and their optional potential for mosquito control is well recognized [1]. Cyclopoid copepods play an important role in aquatic food as well as either primary consumers or predators. They are intermediate hosts of many parasitic worms that are associated with human health. Hence the study on their complete biology is having significance in terms of aquaculture economy and also human welfare.

The copepods present in the Indian waters have been well studied taxonomically, but not biologically [2]. Few reports were available regarding their feeding [3] behavioural [4] and reproductive biology [2, 5].

However, to the best of our knowledge, no studies pertaining to the isolation and identification of hemocytes of copepods are available. Hence the present study has been focused on developing a methodology for isolation of hemolymph and hemocytes from there microscopic organisms and to identify the circulating hemocytes. Hemocytes are multipurpose host defense cells in invertebrates. Crustaceans display relatively simple hemocytes [6] that are mainly subdivided into three cell types hyaline, semigranular and granular. They differ in their morphology, biochemical characteristics and functions [7]. In the present study, an attempt was made to give a first report of hemocytes in the cyclopoid copepod in freshwater pond ecosystem.

MATERIALS AND METHODS

Plankton Collection

Zooplankton species were collected by Towing method using Henson's standard plankton net (150 μm mesh) in zigzag fashion horizontally at a depth of 50 to 100 cm for about 10 minutes with a uniform speed, in the freshwater pond (Mathi Pond) located in Kumbakonam (Lat.10.9617° N, Long. 79.3881° E), Thanjavur district, Tamil Nadu. From the zooplankton population collected,

the cyclopoid copepods were isolated and cultured separately.

Isolation Hemolymph, Plasma and Hemocytes

20 mature adults were separated under a hand lens. Using a tissue homogenizer the planktons were crushed using a physiological saline solution. The homogenate was then centrifuged in 3000 rpm for 10min. the tissue debris was discarded. The supernatant was supposed to be the hemolymph. To centrifuge 6000 rpm for 10 min this resulted in the separation of plasma and packed cells out of the hemolymph. The plasma was separated in an Eppendorf tube using a micropipette. The packed hemocytes were re-suspended in phosphate buffer saline for further identification.

Microscopic Examination of Hemocytes

Freshly packed hemocytes stained with eosin were examined by light Microscopy (LM) to determine the cell size, cell shape and presence of granules under a magnification of 100X. A calibrated micrometer was used for cell measurements.

Our results suggest that the hemocytes of cyclopoid copepod are composed of three major groups, hyaline cells, semi granulocytes and granulocytes, which have distinct morphological differences. Most investigators have historically recognized these three categories and separated those using morphological criteria [8]. Based upon results with Eosin staining, the zooplankton hemocyte cells were classified according to size, shape and presence or absence of cytoplasmic granules. (i) Hyaline cells: Cells with no evidence of cytoplasmic granules. These are found in abundant (Figure 1). (ii) Semi granulocytes: Cells with the variable number of cytoplasmic granules. (iii) Granulocytes cells with a great number of cytoplasmic granules (Figure 2A to D).

Summarizing, light microscopy analyses performed in the present study allowed us to identify three main hemocyte types in hemolymph from the zooplankton cyclopoid copepod. Although the preliminary morphological investigation of first report hemocytes under the light microscope is considered necessary by investigators, ultrastructural and functional studies are also needed to better distinguish crustacean cell types and clarify their role in immune responses. Further studies by investigators utilizing other zooplanktonic species are necessary to test the usefulness of this classification scheme and to offer improvements by developing more specific criteria.

Fig. 1: *Light microscopic observation of Mesocyclops pehpeiensis (Cyclopoid Copepod).*

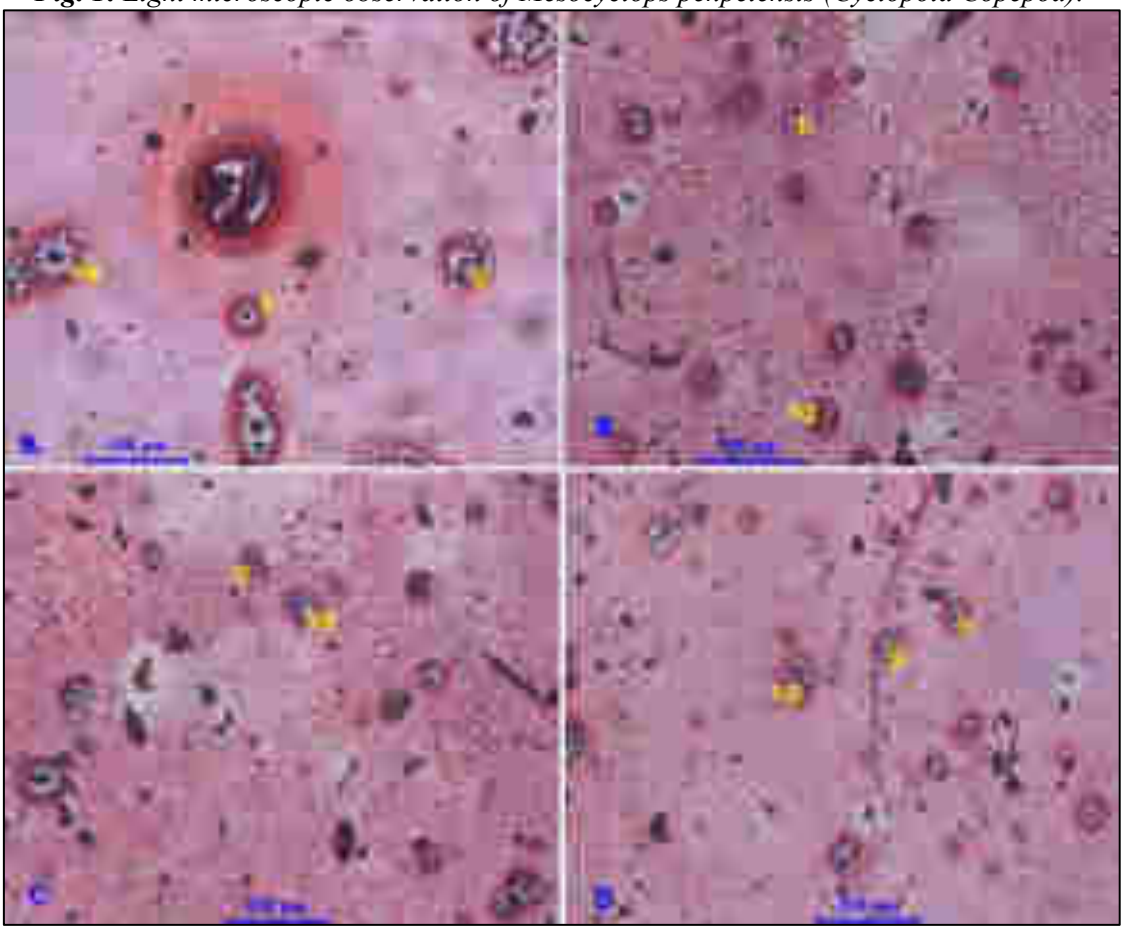

Fig. 2 (A to D): *Cyclopoid copepod haemocytes stained with Eosin (2% w/v) observed in vivo. g: granulocytes; sg: semigranulocytes; h: hyalinocytes. Bar length 100µm, 200 µm and 500 µm.*

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest to publish this manuscript.

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Haematological variables of *Oreochromis mossambicus* against *Aeromonas hydrophila* infection by using dissimilar types of gaumutra distillate

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In Aquafarming, gaumutra has been used to recuperate broad healthiness of a specific person. An analysis was conceded out to assess the consequence of different breeds of cow and bull urine (distillate) was used for the analysis of haematological parameters of *Oreochromis mossambicus* fish against *Aeromonas hydrophila* bacterial infection. Average weight (15.6 ± 0.2 g) of healthy fishes was maintained in 70 litres aquaria with most favourable temperature range from 25.8 ± 28.8 ^{o}C. Furthermore, fishes were exposed to different cow urine distillate (CUD) namely, C (without cow urine), T1 (Gir calf), T2 (Gir cow), T3 (Gir Bull calf) and T4 (Gir Bull) on 0.1 % concentration in the medium for seven days. *O. mossambicus* injected with heat-killed *A. hydrophila* (1×10^{-1} cells) during post stimulation phase. Meanwhile, blood samples were collected to determine the different haematological parameters *viz*. total RBCs, WBCs, haemoglobin concentration and mean corpuscular haemoglobin. One-way analysis of variance exhibited significant value ($P<0.01$) of haematological parameters and as well as haematological contents. Based on the above findings, the present study revealed that T2 (Gir cow) urine distillate has the potential antimicrobial activity of *A. hydrophila* and also enhanced the health status of *O. mossambicus*.

[**Keywords:** *Aeromonas hydrophila*; Cow urine; haematology; *Oreochromis mossambicus*; T2- Gir cow]

Introduction

Aquafarming is a unique fast upward nourishment's creating sectors of the universe and intended to enlarge productivity per unit space. Fish farming is considered as an outstanding innovation in the use of organic waste. The manure (cow urine) and supplementary feeding play an imperative role in various types of fish farming practices. The feed and fertilizers cost could be reduced significantly by integrating fish farming with livestock farming which ultimately is economical with respect to the cost of production[1]. The augmented escalation of aquaculture has managed a great number of pathogens, the major cause of the outbreak of diseases in fishes. Therefore, distant predictable methods such as the use of disinfectants and antibacterial medications have been inadequate achievement in the avoidance or therapy of fish infections. The enormous use of antimicrobials for disease resistor and development promotion in fish proliferations the selective pressure used on the microbial world and boosts the usual emergence of bacterial resistance[2]. The use of inoculations in fish farming to avert the bacterial diseases is successful[3], but the main safeguard is species (pathogens) specific and expensive[4].

Recently, cow urine has been reported as a bio enhancer source of allopathic antibiotics and anticancer drugs[1]. It enhanced their effect and reduced the toxic substances and others including adverse effects of their synthetic drugs. As per Ayurveda, cow urine is needed to purify and detoxify in many drugs. Cow urine distillate (CUD) known as "Kamadhenu ark", exhibited many biological activities including immune modulatory and anti-potential anti-microbial effect of various living beings[5]. Freshwater fish *Oreochromis mossambicus* (Tilapia) is often used as a good experimental model and is extensively used in biological, genetic and physiological studies in relation to pollution, stress, or growth promoters[6,7]. Tilapia is an excellent experimental model for haematological studies and microbial infection because of its worldwide economic importance and also tolerates poor water quality conditions. Therefore, the current work was intended to examine the haematological changes and antimicrobial effect of cow urine distillate of T2 (Gir cow) in *Oreochromis mossambicus*.

Materials and Methods

Acclimatization

Oreochromis mossambicus weighing (15.6 ± 0.2 g) and body length (7.8 ± 0.2 cm) of both male and female fishes were bought from the local fish farm, Kumbakonam (Lat. 10.9617° N, Long. 79.3881° E), Thanjavur district, Tamil Nadu. Fishes were carried to the wet lab and adapted for 1 week prior to experimentation. Plastic tubs were washed and then sundried to avoid fungal contamination. Healthy fishes were transferred to plastic tubs (30 liters) containing dechlorinated tap water. They were regularly fed with formulated food and the medium was changed daily to remove faeces and food bits and pieces.

Collection of cow urine

Six disease free cow of each breeds were designated for urine collection. The initial sunrise (4 to 5 am) first urine of Gir calf and Gir bull calf (2 years old) Gir cow and Gir bull (6 years old) was collected from three different sampling stations namely, Goshala, Sri Vittal Rukmini Samsthan, Govindhapuram near Kumbakonam. All cow's urine was collected separately and carried to the lab in sealed antiseptic containers following the methodology of Sattanathan et al[8].

Preparation of cow urine distillate

Dissimilar sex breed Gir goumutra (cow urine) was distillated at 50-60 °C and exhausted by glass multiple distillation apparatus[9].

Experimental setup

After two weeks of acclimatization four groups of fish were treated, each with Gir cow-calf (T_1), Gir cow (T_2), Gir bull calf (T_3) and Gir bull (T4) cow urine distillate at 0.1 % concentration, respectively. A control group was maintained separately without cow urine treatment for seven days.

Preparation of heat-killed whole cell vaccine

A solo colony of *A. hydrophila* from the agar plate was inoculated in the tryptic soy broth. After 24 hrs, the bacterial cells in the broth were exposed to 60 °C for 1 hour in a water bath. The sterility was tested by inoculating a sample on nutrient agar plates. The heat-killed bacterial culture was centrifuged at 3000 rpm for 15 minutes. The supernatant was discarded and the pellet was re-suspended in sterile Phosphate Buffer Saline (PBS). Then the bacterial number was calculated by measuring optical density (OD) in a spectrophotometer and also ascertained by plate count method. The final bacterial concentration was adjusted to 1 x 10^7 cells/ml by serial dilution[10]. The experimental fishes were tested intra peritoneally with the bacterial suspension of 0.2 ml.

Serial bleeding

The Pisces were bled consecutively using 1 ml tuberculin syringe (Glass van) with the 26-gauge syringe from the common cardinal vein situated just below the gills, at systematic intermissions of 7 days after immunization[11]. The blood drawn was collected in heparinized haematological tubes. Great attention was engaged to evade foaming when drawing the plasma into micropipette as this readily resulted in haemolysis[12].

Collection of blood

About 4 -5 ml of blood sample was collected from the fish to use single heparinized one-use syringes containing 0.5 mg ethylene diamine tetra acetic acid (EDTA) as an anticoagulant; correctly mixed and stored at -20 °C for haematological analysis. The blood was stored in -4 °C in the deep freezer prior to analysis.

Blood cell count

The red blood corpuscles (RBC) and white blood corpuscles (WBC) were calculated using haemocyto-meter crystalline chamber using "Hayem's" and "Turch's" diluting fluid, respectively.

Statistical analysis

One-way analysis of variance was executed using Minitab (Version 11: SPPS Inc. Chicago, Illinios USA) software for investigating the significance between mean (MS Excel Microsoft office 2007) was used for graphical presentation of data.

Results and discussion

The changes of haematological parameters like, RBC, WBC, hemoglobin (Hb) and mean corpuscular hemoglobin (MCH) in the fish *Oreochromis mossambicus* both in control were analyzed and also the surveillance rate exposed after the 28th day of the post-immunization process was studied.

Haemoglobin content

Haemoglobin content varied between 5.2 to 7.7 g/dL. The minimum haemoglobin content (5.2 g/dL) was observed in control and the maximum

(7.7 g/dL) content was observed in T3 (Gir bull) (Fig. 1). The response reached its maximum on 14th-day post-immunization as the peak day. Similar findings were reported by Sakthivel[13]. Decreased hematocrit and haemoglobin absorption designate that RBC are being demolished by the leucocytosis action in an erythrocytic anaemia with subsequent erythroblastosis[14]. An increase in hematocrit has been stated as an outcome of oxygen deficiency[15].

Total erythrocyte counts

The total erythrocyte counts of blood sample of the T3 group was found to be the higher and fluctuated significantly ($p < 0.001$) with additional treatment sets. The maximum value of 14.2 $x10^6$ cells/cu mm^{-3} erythrocytes count was observed in T3 and minimum of 11.20$x10^6$ cells/cu mm^{-3} erythrocyte count was observed in control (Fig. 2). Ascribed the reduction in the RBC to heamolytic crisis that effects in cautious anemia in Pisces exposed to heavy metals and herbicides respectively[16,17]. Moreover, the decrease of RBC also primes to growth of hypoxic state which in turn leads to growth in damage of RBC or less in rate of formation of RBC due to non-obtainability of Hb content in cellular medium[18]. The peak day was found to be on the 21st day of post-immunization.

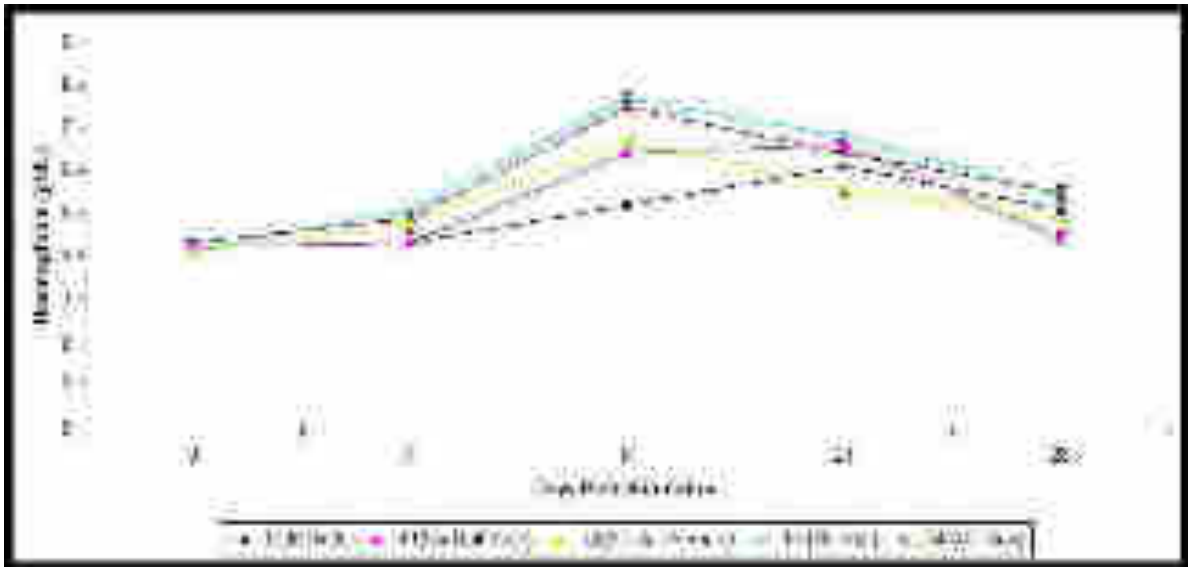

Fig. 1 — Haemoglobin content in *Oreochromis mossambicus* on exposed to dissimilar varieties of gaumutra distillate

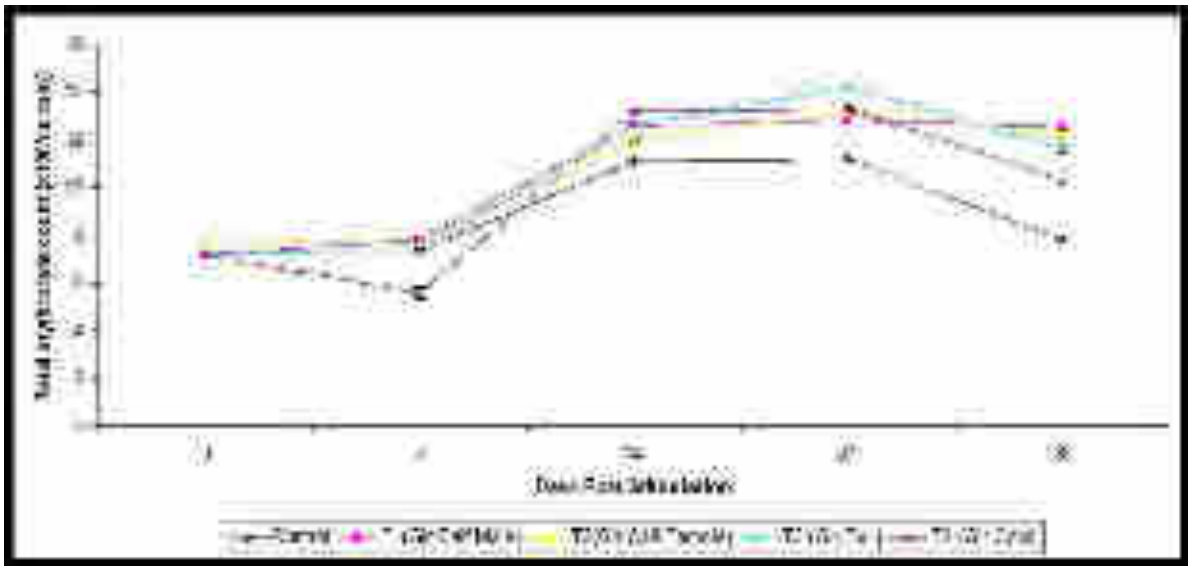

Fig. 2 — Total erythrocyte counts in *Oreochromis mossambicus* on exposed to dissimilar varieties of gaumutra distillate

Mean corpuscular hemoglobin (MCH)

MCH rate of investigational sets was created to diverge expressively ($p>0.05$) when related to control sample (Fig. 3). The blood indices such as MCH, MCV and Mean corpuscular haemoglobin concentration (MCHC) are mainly vital for the finding of anemia in utmost animals[19]. The maximum value was observed in T4 (8.41 %) and the minimum value was observed in T1 (5.96 %).

Total leukocyte counts

Total leuckocyte cells were originated to be the higher value in T2 when compared expressively ($p<0.001$) by control groups T1, T3, and T4. The highest leukocyte counts of $29.6x10^3$/cells cu mm^{-3} were noticed in T2 groups and lowest value of $10.9 x10^3$/cells cu mm^{-3} was noted in control (Fig. 4). Reaction of the defence mechanism of the fish by leucocytosis under pathological conditions and against foreign bodies[20]. A highest activity was found on the 21st daytime of post-immunization.

Relative percentage survival rate

In the current investigation, goumutra of different breeds condensed the death of fishes tested with an infectious strain of *A. hydrophila*. Comparative percentage of existence rate is decreased by altogether treatments (Fig. 5). Control was observed with 66 %, T3 and T4 with 50 % and 75 % protection, respectively while T2 conferred 33 % protection. The measurement of haematological parameters, which are used in this investigation, has provided valuable

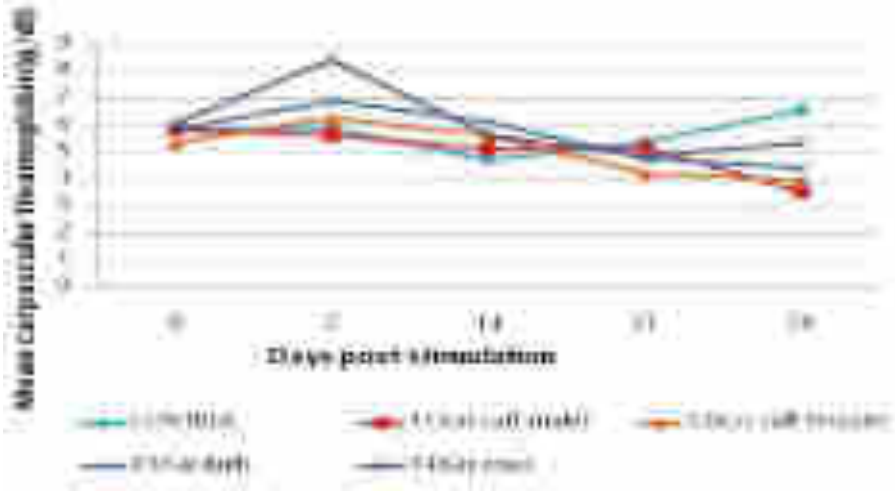

Fig. 3 — Mean corpuscular Haemoglobin content in *Oreochromis mossambicus* on exposed to dissimilar varieties of gaumutra distillate

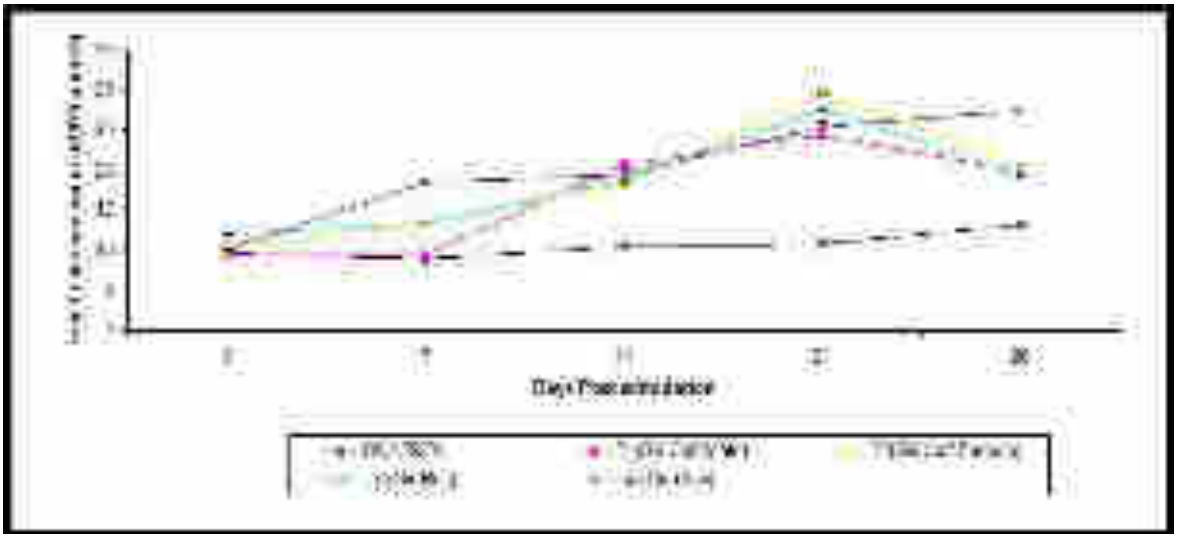

Fig. 4 — Total leukocyte counts in *Oreochromis mossambicus* on exposed to dissimilar varieties of gaumutra distillate

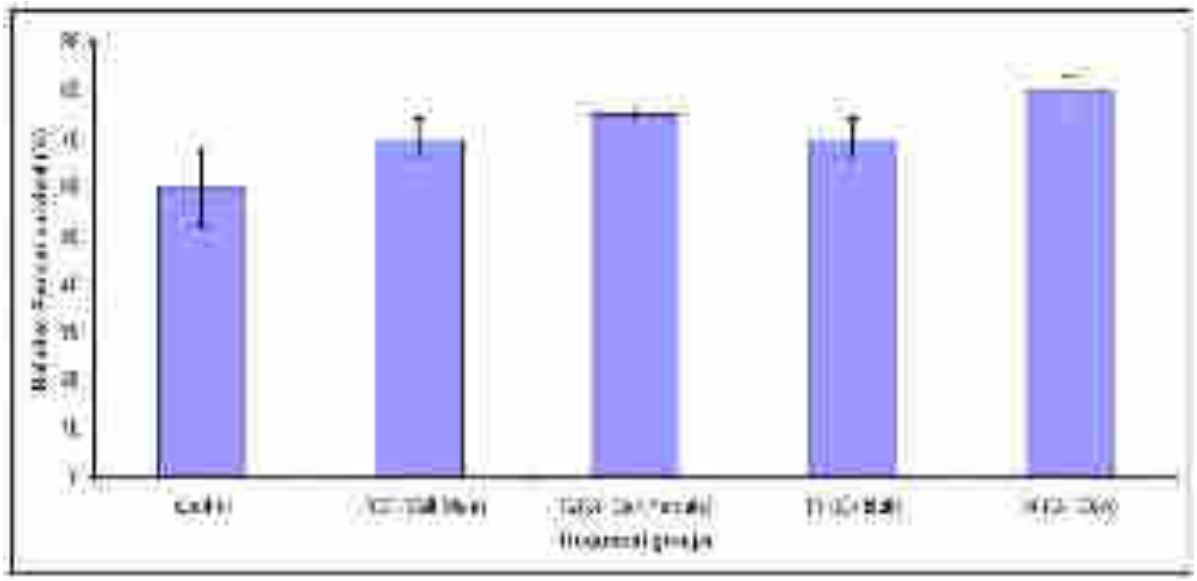

Fig. 5 — Relative percentage survival rate (%) in *Oreochromis mossambicus* on exposed to dissimilar varieties of gaumutra distillate

information which can contribute to the applied and basic research needs of an aquatic field in the assessment of fish health status and in monitoring stress responses like immunization with heat hilled *A. hydrophila*.

At present, tiny is well-known about the haematology of the tilapia *O. mossambicus*, which is unique of the most vital organism in freshwater aquaculture ecosystem; the outcomes accessible above have exposed a stimulating strategy of response on the haematological difference in strained fish. When we compared *O. mossambicus* normal blood standards with those acquired earlier for this species, we found less values of haematocrit (20 %) and RBC count (1.31 x10^6 cells/ µL)[21] but obtained somewhat greater values for haemoglobin concentration (6 g/dL). Hrubec et al.[22] Reported even higher haematocrit (33%), RBC count (2.31x10^6 cells/ µL) and haemoglobin concentration (8.2 g/dL). In almost all infected fishes, the homeostatic procedures are prolonged beyond the regular limits due to stress[23]. These changes may be due to various strains used in earlier studies and diverse environment and culture settings.

In the fish inserted with the heterotrophic gram-negative bacteria that causes more death in tilapia culture structures, the investigational inoculation only triggered a subclinical contagion and no clinical marks of an infective process were noticed. In agreement through the observation of the corpuscular persistent and the perceive results in the erythrocytes and leukocytes count of blood smears. Similar findings were reported by[24] described microcytic hypo chromic anemia in *Paralichthys olivaceus* due to the reduction of mean corpuscular counts, hypoglobulia and the structural irregularity of the erythrocytes. Subsequently, numerous animals have shown to improve the growth parameters, length, weight, specific growth rate and survival rate in *O. Mossambicus*[25].

Our findings suggest that cultured *O. mossambicus* in cow urine distillate (CUD) against *A. hydrophila* showed significantly ($p<0.001$) improved growth performance when compared with control.

Acknowledgement

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Efficacy Of Cow Urine Distillate To Enhance The Freshwater Copepod Population Density

Praveena Varadhan, Subhhasri Varadharajan, Priya Muthukirushnan, Venkatalakshmi Sournamanikam

Abstract--Zooplankton has high nutrition value because of some vitamins, amino acids and fatty acids in them. Cow urine is known for its medicinal properties and therapeutic value in india. There are many reports which revealed the application of cow urine in agriculture, poultry, animal health and human health. However, there are no reports for its application in live feed culture. Hence, in the present investigation, the effect of Gir Cow Urine (CUD) was studied on zooplankton for it's impact on biomass and biochemical composition. The study revealed that the biomass of zooplankton significantly increased in Gir cow urine distillate treated group. The experiment was carried out one month period. At the end of study period, the biochemical composition of cow urine treated and untreated zooplankton cultures were analyzed.

Key Words--Zooplankton, Gir , Cow urine distillate, Aquaculture, Biochemical analysis, Live feed

1. INTRODUCTION

The word "Plankton" derived from Greek mean drifters and the word was first coined by Victor Hensen in 1887. Plankton commonly falls under two categories namely Phytoplankton and Zooplankton. Phytoplankton are primary producers and minute photosynthetic plants. Zooplankton are secondary producers and animal groups of the freshwater ecosystem. Copeopods and Rotifers are the largest, most diversified group in freshwater ecosystem. Copepods are considered as ecological indicators of water quality and global climate change. Alterations in their presence and abundance can be resulted in the low fisheries production. As the major secondary producers of the ecosystem, they fed on phytoplankton and act as food for many major commercially important fishes. For example, a herring stomach contains 60,000 calanoid copepods (1). The studies on copepod ecology in different habitats like estuaries, mangroves, seagrass beds, seaweeds, coral reefs, lagoons and open seas is important to understand the ecosystem health and fish production. Copepod is one of the most abundant live feed organisms which are widely present in almost all the freshwater bodies. Copepods pass through very distinct life stages. They emerge from an egg as a nauplius, usually 100-150uM in length. After six nauplius stages (referred as stages N1 to N6), with growth between each stage, the body shape changes and a series of usually six copepodid stages follow (referred to as stages C1 to C6).

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The last of these stages is the adult in which different sexes can be identified. Copepods used as natural food are either cultured or collected from natural water bodies (2) Freshwater finfish seed production often faces a problem of an in adequate food supply. Artificial feeds are widely used, but planktonic animals are very important, especially rotifers, cladocerans, and copepods. Virtually all fish feed on plankton, especially in their early life phases. Planktivorous fish depend on small invertebrates throughout their entire lives. Copepods of the order Cyclopoida are the most important food items in freshwater aquaculture, and their nauplii are especially valuable for feeding fry (3). The importance on mass culture of copepods begins due to the unsatisfactory performance of traditional live feeds Artemia and Rotifer in larviculture. In mangroves and estuarine environment, harpacticoid copepods are the major food for larvae and juveniles (4). The key problem in culture of copepods is high yield in the sense of hatchery level on a continuous basis. Depletion of DHA (Decosahexanoic acid) and EPA (Ecosapentanoic acid) in the food of the larvae leads to low survival, structural deformities and malpigmentation (5). So that fish and larvae rely mainly on the live feeds which contain high nutrition. The medicinal usage of cow urine is practiced in India from ancient days. Hence cow urine could be expected as a good immunostimulant and water quality enhancer. Cow urine contains various inorganic compounds including silver, Na-K ratio of 4:1 (36%:9% in dried urine), apart from about 3% urea. Fresh cow urine also contains 50-100 mg oestrogens/100 ml; 20- 200 µg of cortico-steroids/100 ml and 0.05-0.15 mg of 17-keto-steroids/100 ml (6). Now days, a lot of emphasis has been given on the medicinal use of cow urine in India. Recently the cow urine has been granted U.S. Patents (No. 6,896,907 and 6,410,059) for its medicinal properties, particularly for its use along with antibiotics for the control of bacterial

infection and fight against cancers (7). A number of diseases could be cured by the use of medicines derived from the cow. Though it is a cheap resource, its benefits are very high. Cow urine contents are 95% water, 2.5% urea and 2.5% minerals, salts, hormones, and enzymes. *Gomutra* alone has got all such chemical properties, potentialities and constituents that are capable of removing all the ill effects and imbalances in the body (8). The laboratory analysis of cow urine shows that it contains nitrogen, sulphur, phosphate, sodium, manganese, carbolic acid, iron, silicon, chlorine, magnesium, citric, succinic, calcium salts, Vitamins A, B, D, E minerals, lactose, enzymes, creatinine, hormones and gold acids (9). Cow urine meets the deficiency of these micronutrients in the body and maintains the balance of these substances and cure seven the so called incurable diseases (10). The presence of gold As aurum hydroxide is recently proved by scientists of Junagadh University (11). Hence the attempt to apply cow urine in aquaculture will bring an integrated farming practice and will have interdisciplinary relevance. Recently, cow urine and its distillate has been examined for their effect on growth promotion in aquaculture *Cirrhinusmirgala* (Hamilton) (12); growth and food utilization parameters of *Labeo rohita* (13). Cow urine distillate known as 'Kamdhenuark' exhibited many biological activities including immunomodulatory potential (14) and antimicrobial effect (15). So in the present investigation, CUD (Cow Urine Distillate) was considered for its application in aquaculture for commercial production of live feeds.

2. MATERIALS AND METHODS

2.1 Sampling area

Sandhana pond (Latitude, 11.09° and Longitude, 79.37°) at Veppathur village, Kumbakonam taluk, Thanjavur district was selected for the present study. It is a perennial pond potential for intensive fresh water aquaculture. However, it has not yet been used for any productive purposes and only being polluted by human interferences. Hence the pond has been selected as the site for study to check the possibility for aquaculture practices.

2.2 Plankton Collection

The plankton and water samples were collected from selected habitat during morning hours (5.00 A.M to 6A.M) by towing method using Henson's standard plankton net (150µm mesh) in zigzag fashion horizontally at a depth of 50 to 100 cm

2.3 Isolation and identification of copepods

The collected samples were immediately transported to the laboratory by providing aeration using battery aerator. The zooplankton samples were thoroughly rinsed to reduce the contamination from other zooplankters. From the samples, Cyclopoid copepod were identified under microscope. The identification of plankton was made by referring the standard manuals, text books and monographs (16-20). Photomicrographs were taken using Trinocular Microscope (Model BXL) attached to an USB camera (MIDCE-5C). Based on the key provided by the authors the species was confirmed for their taxonomy and used for culture.

2.4 Cow urine collection

Six disease free cows were selected for urine collection (Tag. No: 0206, 0177, 0184, 0468, 0133, 0201), were collected from Goshala, Sri Vittal Rukminni Samsthan, Govindapuram near Kumbakonam. These animals were maintained in a ventilated shed with the provision of individual feeding and watering. Clean drinking water and feed was provided *ad libitum.* Animals were daily offered about 2 kg of feed and the available green fodder. The early morning first urine (4.00 to 5.00 am) was collected from six cows it was pooled and transported to laboratory in airtight sterile containers.

2.5 CUD preparation

The collected urine samples were distilled simultaneously at 50° C - 60° C using distillation apparatus for 5 – 6 hours (21). The cow urine distillate (CUD) was stored in sterile glass containers and was used for treatment on the same day without storage (22).

2.6 Developing pure culture of Cyclopoid copepod

To begin with, *Cyclopoid sp.* collected from the study pond was maintained in 2 liter capacity containers, provided with aeration. The *Cyclopoid sp.* was identified, under microscope using the 'Practical Guide to Identify Freshwater Crustacean Zooplankton' (23). The Cyclopoid species were isolated and inoculated in to another 2 liter container and fed with yeast media. The yeast was dissolved in clean and filtered freshwater to avoid dust particles and other waste materials. Feeding was done once in three days or whenever the water in the culture tank becomes clear. Excess feed would cause water quality problems which would lead to the mortality of copepods; hence care was taken to avoid excess feeding.

2.7 Experimental Protocol

Four tanks with twenty liters of stock culture was inoculated with a population density of 500 Nos each. The zooplankton was fed with 2.5ml of yeast stock solution for every three days. The CUD was added in different concentrations viz, 0.025%, 0.05% and 0.1% in three experimental tanks. One tank was kept as control by adding equal volume tap water without CUD. The media is kept undisturbed for a period of seven days. After seven days water was changed. The plankton were well aerated with all culture requirements for 30days. After 30days, the survival, growth and biochemical composition of the CUD treated and untreated plankton groups were carried out.

2.8 Assessment of Growth

To determine the population density 500 adult animal were kept in 20Lit aquarium. The copepods were fed once in every two days. No copepods were removed from the containers during experimental period of 30 days. On final day, all the copepods including nauplii, copepodites and adults were filtered through 48µm sieve. The total copepods produced over the period of experiment were counted using Sedgwick rafter counter under the microscope. The number of nauplii, copepodites and adult copepods were counted separately. The animals were count in 1ml at 5 times and the results were multiply into l lit.

2.7 Biochemical analysis

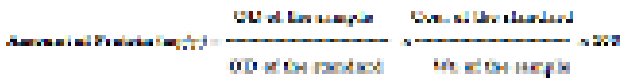
The plankton collected was filtered through plankton net made up of bolting silk pore size 150µm. The filtered plankton was collected for biochemical analysis on wet weight basis.

Carbohydrate analysis

Total carbohydrate was determined by Anthrone method (24). Freshly weighed 100mg of animal were homogenized in distilled water (5ml) and 5% tricholoro acetic acid in a homogenizer. The homogenate was centrifuged at 2500rpm for 5 minutes. To every 1 ml of the supernatant 4 ml of anthrone reagent was added. A standard glucose solution was also run along with the samples. The samples were kept in water bath for 10min at 100°C. The optical density was read in a UV spectrophotometer at 620 nm. The percentage of carbohydrate content of the plankton was calculated using the OD of the unknown sample and that of standard glucose solution. Standard curve was drawn.

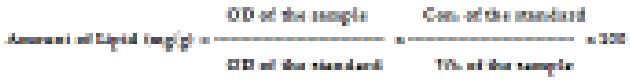
Protein analysis

Protein was estimated following Lowry *et al*., (25) method. Fresh wet weight (100mg) of plankton was homogenized with 5% tricholoroacetic acid in homogenizer. The homogenate was centrifuged at 3000 rpm for 10 minutes and the residue was dissolved in 0.1N NaOH. Exactly 0.2ml of this solution was made up 0.1ml using 0.1N NaOH To this 3.5 ml of Folin's reagent was added and thoroughly mixed. The optical density was measured at 670 nm in UV spectrometer.



Lipid analysis

Lipids were extracted as described by Folch *et al*., (26), and estimation was done by the phospho vanillin method of Barnes and Blackstock (1973). 50mg plankton were homogenized (5% w/v) in a waring blender in chloroform-methanol mixture (2:1). The homogenate was filtered through Whatmann No.1 filter paper and the residue was removed by shaking vigorously with 0.88% KCI (added as one fourth of the volume) 1ml of filtered was taken in a test tube and evaporated under water bath (10 minutes) and 1ml of concentrated H_2SO_4 was added and boiled for 10 minutes. For estimation of total lipid, 0.2 ml of this solution was taken and 5ml of vanillin reagent was added. The developed colour was read in UV Spectrophotometer at 520 nm against reagent blank. Cholestrol was used as a standard.



3. RESULTS

3.1 Population density

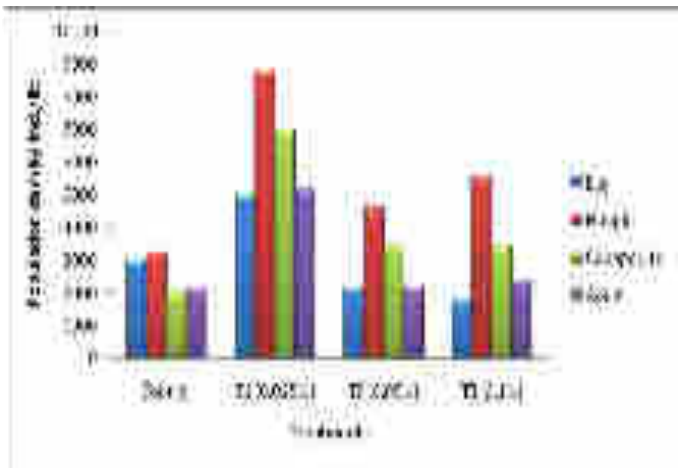


Fig 1: Effect of Gir CUD treatment on Population density of Cyclopoid Copepod

Fig 1, illustrates the effect of different concentration of CUD treatment on copepod population density. The results suggested that, the T2 group which was treated with 0.05% of CUD for seven days showed the maximum response with highest number of nauplii (8800), copepodite (7000) and adult (5200). Whereas in T2 which was treated with 0.025% of CUD, the growth response of population is lower than the T1 group and T3 which was treated with 0.1% of CUD. The statistical analysis showed significance variation ($P<0.05$) among the treatment groups. When compared to control group, all the treatment groups showed higher population density. In all the experimental groups including control and treatment, the number of nauplii, is higher than the copepodites which is tow in higher than the adult.

3.2 Biochemical analysis

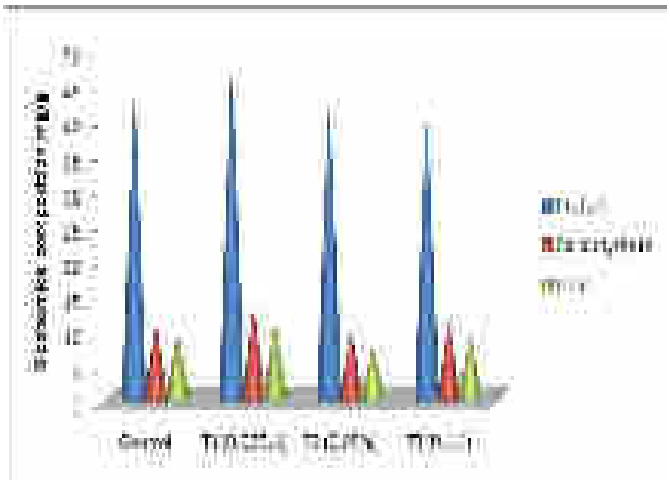


Fig 2: Nutritional Value of Cyclopoid Copepod treated with Gir CUD

(i) Protein content

Protein content of zooplanktonwas estimated on wet weight basis. The findings of the present study as shown Fig. 9 in revealed that maximum protein content of was recorded in T2 group which was treated with 0.05% concentration of Gir CUD for seven days. Minimum protein content of was recorded in T3 for which was treated with 0.1% concentration of CUD. The treatment and control groups showed significant variation ($P<0.05$).

(ii) Carbohydrate content

The amount of Carbohydrate in zooplankton was estimated on wet weight basis. The carbohydrate content was noted as highest (12%) in T2 which was treated with 0.05% concentration of Gir CUD and least carbohydrate content of (9%) was recorded in T1which was treated with 0.025% concentration of CUD (Fig. 9).

(iii) Lipid content

As exhibited in Fig, the result shows that lipid content of zooplankton was significantly influenced by period of cow urine distillate exposure. Maximum lipid content of was recorded in T2 (10%). Minimum lipid content of (7%) was recorded in T1.

4. DISCUSSION

From Ancient Indian Vedic literature it has been known that cow urine has multiple, properties (27) and because of that cow urine has been described to have many therapeutic and spiritual cleansing qualities (28).Wealthy cow excretes a volume of 17-45ml/kg/day with specific gravity ranging from 1.025-1.045. Its pH ranges between 7.4 and 8.4 with seasonal variations. Urea nitrogen and total nitrogen varies between 23 – 28ml/kg/day and 40 – 45 ml/kg/day respectively. In addition it also contains Uric acid, calcium iron and other trace elements. Iron helps in RBC production. The body electrolytes sodium and potassium helps in maintaining new health. The arum hydroxide (gold particles) have been proved to be present in list cow urine by Junagadh Agricultural University Scientists. It acts as antimicrobial anti dose and immunostimulant agent. Recently the practice of using cow urine as therapeutics is formed as cowpathy. Though it has lot of disputes, the clinical properties of cow urine is scientifical validated by various authors (29). The application of cow urine in aquaculture as fresh cow urine (30-31) Cow urine Distillate (32) and also herbal cow urine extracts (33) have been explored in different fish models like *Cirrhinus mirgala, Labeo rohita* and *Oreochromis mossambicus.* The optimal concentration of CUD for promoting growth and biochemical composition is well documented Padmapriya and venaktalskhmi, 2014 for fishes. However the optimal concentration of cow urine distillate has not yet been established for zooplankton. Hence the present study has been attempted to find the optimal concentration of CUD to be treated for copepod culture. From the results obtained, it is clear that 0.05% of CUD is the optimal concentration for plankton growth. This is in concentration with the results obtained for fish studies which showed 0.1% of CUD as the optimal concentration. This might be due to the difference in the body mass. Due to the higher body mass and complexity of the physiological systems, the fishes require 0.1% concentration of CUD to show maximum response. Whereas the copepods, which are micro crustaceans with less evolutionary development of physiological systems need only lesser quantity of CUD for exhibiting maximum response. The CUD shows a dose dependent effect on the population density of copepods. The lower concentration has lesser effect and higher concentration i.e 0.05% shows higher effect. However the 0.1% shows a suppressive effect. This might be due to the fact that CUD also shows toxicity at higher concentration. The mortality of fishes at 5% and 1% concentration was already reported by Sattanathan and Venkatalakshmi, 2015; Padmapriya and Venkatalakshmi, 2014. However there is no documentation for LC50 values of cow urine or CUD on fishes or Planktons. The general pattern of high nauplii count in all the treatment groups reveals that cow urine distillate enhances the fertility rate of copepods. This observation is the first of its kind in the literature of cow urine beneficial properties. Hence this is a good indication that Gir CUD could be a better stimulant for copepod culture and could be strongly considered for mass culture. Similarly the high protein count of the isolated species in Sandhana pond of Veppathur village confirm the high nutritive value of copepods as live feed in aquaculture. Hence the present study falls in live with the previous findings of this laboratory that CUD is a good natural growth promoter and has provide scopes in the field of ecosafe organic aquaculture.

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Original article

Identification of a novel antibacterial protein from hemolymph of freshwater zooplankton *Mesocyclops leuckarti*

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ABSTRACT

Bacterial infections are the most important problem of health care worldwide. The hemolymph antibacterial proteins of *Mesocyclops leuckarti* was isolated for the first time and its antibacterial efficacy was evaluated against four different human pathogenic microbes viz., *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumonia* and *Shigella flexneri*. The antibacterial potential of the antimicrobial proteins of hemolymph samples from plankton cultured in water enriched with Cow Urine Distillate (CUD) was compared with normal ones. The results indicated that the hemolymph proteins were more potential against Gram negative bacteria than Gram positive bacteria. *Klebsiella pneumonia* was more susceptible to the hemolymph proteins exhibiting a zone of inhibition measuring 27 mm. The supplement of CUD to the culture media further enriched the antibacterial activity of the hemolymph proteins (29 mm). The SDS-PAGE analysis indicated two different types of clear bands representing proteins of 53 kDa and 19 kDa. Overall, this investigation signified that the microcrustaceans have a defence mechanism hemolymph of *Mesocyclops leuckarti* have a potential agent for novel antibiotics.

1. Introduction

Copepods represent about 20% of the mean annual zooplankton biomass. They are found in abundance in many aquatic ecosystems (Huys and Boxshall, 1991) and conspicuous member of aquatic biota that have adapted to various habitats such as damp moss, interstitial sand, subterranean localities and parasitic living. The cyclopoid copepods are successful inland water group animals and *Mesocyclops leuckarti* is the important planktonic cyclopoid copepods. In evolution, invertebrates have not developed acquired immune systems. However, they have been bestowed with non-specific immune mechanisms. Crustaceans show many antibacterial proteins in their hemolymph. However, the literature does not show any record of anti bacterial proteins in microcrustaceans including zooplankton (Iskratsch et al., 2009; Iwanaga, 1993; Kawabata et al., 1995; Mori and Stewart, 1978; Jayasankar and Subramoniam, 1999) (Table 1).

Hemolymph is the type of blood found in Arthropod's open circulatory system. It contains many bioactive molecules which have functional roles in the defence system. The molecules include lectins, complement, clotting factors, antimicrobial peptides (Vazquez et al., 2009).

Among them the antimicrobial peptides are the prime factors that give immunity to the animal. There are two types of antimicrobial peptides identified in hemolymph. They are high molecular weight large antimicrobial proteins (>100 amino acids) and low molecular weight small antimicrobial proteins. The high molecular weight antimicrobial proteins target the disrupt microbial biomolecules and small antimicrobial proteins disrupt the structure and/or the function of microbial cells (Aspan et al. (1995); Stabili et al. (1999); Fujimoto et al. (1995); Hall et al. (1995)). These antimicrobial peptides are secreted in

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Table 1
Antibacterial activity of high molecular protein in freshwater zooplankton *Mesocyclops leukarti* hemolymph against bacterial pathogens.

Samples	Zone of Inhibition in diameter (mm)			
	Klebsiella pneumonia	*Escherichia coli*	*Shigella flexneri*	*Staphylococcus aureus*
Control	–	–	–	–
S1	27 mm	20 mm	17 mm	19 mm
S2	29 mm	25 mm	20 mm	25 mm
Amikacin (Standard)	27 mm	25 mm	28 mm	30 mm

- = No zone
Control = Distilled water
S1 = Hemolymph of plankton cultured without cow urine distillate
S2 = Hemolymph of plankton cultured with cow urine distillate

hemolymph in response to microbial invasion the structure and or the functions. Similarly, the hemocytes in the hemolymph are called as granulocytes or amoebocytes that contain two types of secretory granules (large and small but dense) Toh et al. (1991). These granules are highly sensitive to bacterial endotoxins. Serine protease zymogens (factor C and factor G) (Muta et al., 1993; Mutaet al., 1993; Mute and Iwanaga,1996) forming the components of granules are auto catalytically activated by LPS and Jayasankar and Subramoniam (1999) β-D glucan present in Gram negative bacteria and fungi and triggers the coagulation cascade. This activation leads to the formation of insoluble coagulin gel from coagulogen and engulf or immobilize the invaded pathogen by forming clot. The entrapped microbe is subsequently killed by lectins and antimicrobial substances released by the granules (Iwanaga, 1993; Iwanaga et al., 2001; Iwanaga et al., 1992).

Recently, exopolysaccharide (Abinaya et al., 2018), selenium nanowires (Abinaya et al., 2019), green symthesized Ag nanoparticles (Jayanthi et al., 2017; Ishwarya et al., 2017; 2019; Al-Ansari., 2019), β-glucan-binding protein (Divya et al., 2020a,2020b), Biopolymer gelatin-coated zinc oxide nanoparticles (Divya et al., 2018), hen's albumen extract (Sherly Carolyn et al., 2019), biopolymer zein-coated gold nanoparticles (Suganya et al., 2017), chitosan-alginate microspheres (Thaya et al., 2018), phytoextracts (Govindarajan et al., 2008; Kolanjinathan et al., 2009) are used for the antibacterial activity.

Though this information is widely available in literature on crustacean immunity, none of the studies has been reported so far on micro crustaceans including Zooplankton. The present investigation was taken up to find proteins from hemolymph of *Mesocyclops leuckarti* and to evaluate their immune potential in terms of antimicrobial activity.

2. Materials and methods

2.1. Collection and maintenance of Mesocyclops leuckarti

Mesocyclops leuckarti were collected from a local freshwater pond in and around Kumbakonam (10.9602° N, 79.3845° E), Thanjavur district, Tamilnadu, India and cultured in our laboratory with 0.025% of cow urine distillate medium.

2.2. Isolation of hemolymph

Using a hand lens 20 mature *Mesocyclops leuckarti* adults were separated and homogenized with sterile physiological saline solution in a tissue homogenizer. The homogenate was centrifuged for 10 min at 600 rpm. The resulting supernatant alone was collected and the cell debris was throughout. The collected supernatant was supposed to be the hemolymph (Praveena and Venkatalakshmi, 2019).

2.3. Estimation of total proteins in hemolymph

Lowry et al. (1951) method with bovine serum albumin as the standard was followed for quantification of proteins in hemolymph samples.

2.4. Molecular weight determination of protein in hemolymph

The purity, homogeneity and molecular weight of proteins obtained from hemolymph of zooplankton, *Mesocyclops leuckarti* was determined by SDS-PAGE consisting of 4% and 12% polyacrylamide in stacking and separating gel respectively. 10 μl of the sample was taken and mixed with 10 μl twofold concentrated buffer. The mixture was heated for 15 min at 70 °C Deraz et al. [2005]. The sample is now loaded in the gel and electrophoresis was carried at a constant voltage of 60 V for two hrs. For determining the molecular weight of the protein bands, molecular mass markers were used. Staining of the gel was done using ethidium bromide.

2.5. In gel digestion

The same process described earlier in followed for digestion process Shevchenko et al. (2007). The gel was placed on a light box and the separated bands were cut separately using a clean sealpel. The excised bands were cut into small cubes measuring 1x1 mm and centrifuged in a micro centrifuge. The gel cubes were washed with destaining buffer (25 mM NH_4HCO_3 dissolved in 50% ethanol) for 20 min at 25° C. The process was repeated until the ethidium bromide stain was taken off. The gel cubes after destaining were dehydrated using 100% acetonitrile for 10 min and rehydrated with 10 mM DTT in 50 mM NH_4HCO_3 (reduction buffer) and incubated for 60 min at 55° C. After incubation the gel cubes were treated with alkylation buffer (50 mM iodoacetamide in 50 mM NH_4HCO_3) and incubated in dark conditions for 45 min at 35° C. Then the gel cubes were washed for 20 min with digestion buffer (50 mM NH_4HCO_3 in H_2O; pH 8.0). Dehydration and washing was repeated continuously for proper drying. The dried gel cubes were coated with trypsin solution and refrigerated at 4° C for 30 min. The gel matrix was further treated with extraction buffer (3% trifluoroacetic acid and 30% acetonitrile) for 15 min to remove the peptides from the gel. After extraction the gels were centrifuged and the supernatant was collected and dried to remove acetonitrile and reacidified with 2% trifluoroacetic acid. The digested peptides were stored at −25° C until further use.

2.6. Antibacterial activity

The antibacterial activity of the hemolymph proteins was determined using the well diffusion method. 25 ml of Muller-Hington agar was poured in petriplates. After optimal temperature is attained, it is inoculated with 0.1 ml of 24 h broth culture of patho-

genic bacteria (*Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Shigella flexneri*) in separate plates. The bacterial inoculums is made to mix uniformly by gentle swirling of the plates. After complete solidification of the agar, three wells of 6 mm diameter were formed using a gel borer. Each well is filled with 50 μl of the protein fraction (low or high molecular weight protein), standard antibiotic amikacin and control was left separately. The inoculated perti plates were then incubated at 30° C for 48hr. The diameter of the inhibition zone was measured using calipers. Each antibacterial assay was performed in triplicate and mean values were reported by McKenna, 2013 and Lila and Tendencia Eleonor, 2004.

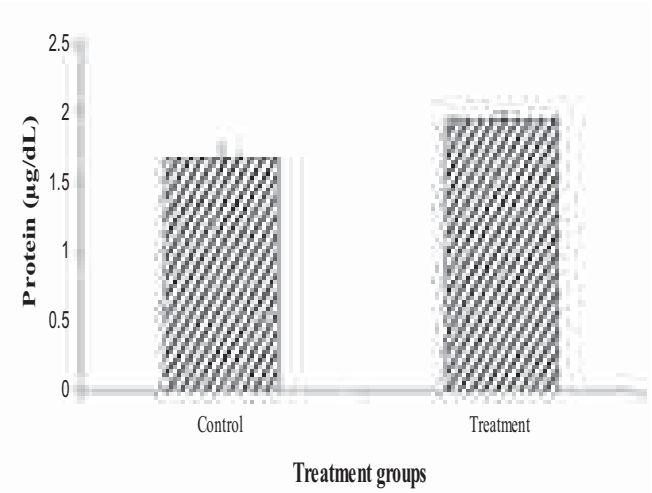
3. Results

The results of fractions by SDS-PAGE indicated the molecular weight of antimicrobial proteins in the hemolymph of *Mesocyclops leuckarti* were approximately in the range between 14 kDa and 100 kDa (Fig. 1). The appearance of single band in each lane in 12% gel indicated their purity.

Fig. 2 depicts the total amount of protein isolated from the hemolymph of the zooplankton *M. leuckarti* that was cultured with the presence and absence of cow urine distillate (CUD) 68 and 96 μg/dl of protein were isolated from the hemolymph of zooplankton cultured with CUD and without CUD respectively (Figs. 3–5).

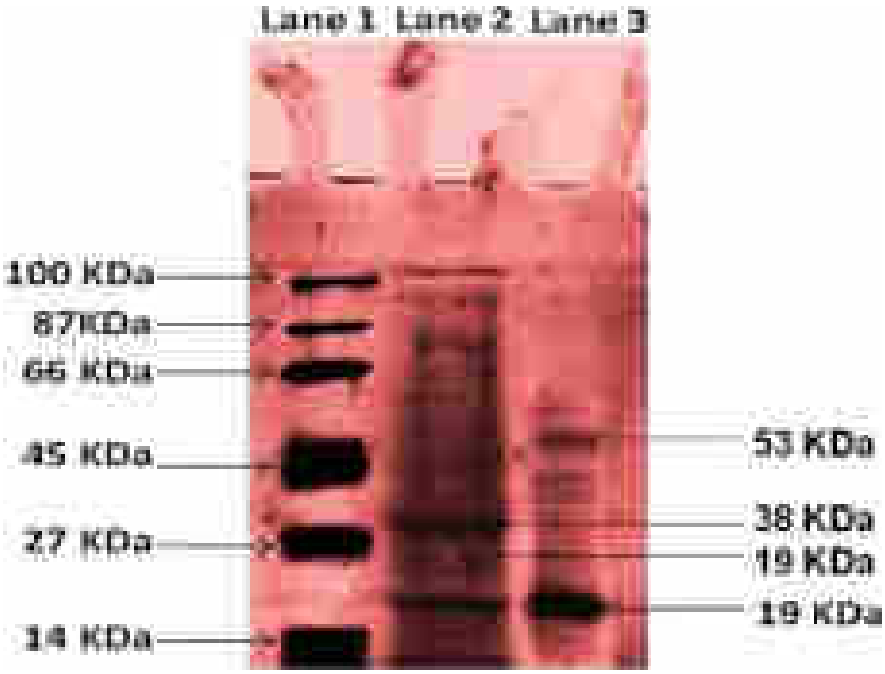
Antibacterial activity of proteins obtained from hemolymph of *M. leuckarti* was assessed by an agar well diffusion method. The results obtained from the experiments conducted are shown in Figures and Tables. From theses results the following facts could be derived. The high molecular weight protein fraction in the hemolymph of *Mesocylops leuckarti* cultured in either in the presence or absence of CUD showed significant antibacterial activity on with the standard antibiotic amikacin against all the human pathogens tested. Among them, the CUD supplementation showed significant impact on the antibacterial activity of the high molecular weight protein fraction of the hemolymph. The maximum activity was shown against *Klebsiella pneumonia* (20 mm) and minimum activity was recorded against *Shigella flexneri* (20 mm) (Figs. 6–8).

The results shown in Table 2, the low molecular weight indicated that the hemolymph samples collected from *Mesocyclops*



Control – Zooplankton culture without Cow urine distillate Treatment – Zooplankton culture with Cow urine distillate

Fig. 2. Protein Estimation of Freshwater Zooplankton Hemolymph and Hemolysate of *Mesocyclops leuckarti*.

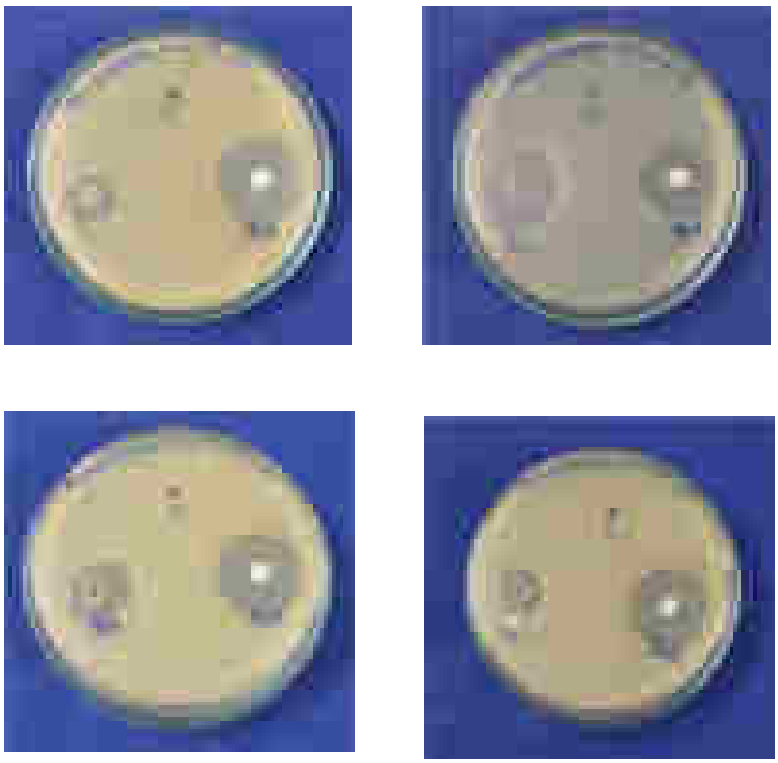


Lane 1 – Marker

Lane 2 – S1 (Hemolymph of plankton cultured without cow urine distillate)

Lane 3 – S2 (Hemolymph of plankton culture with cow urine distillate)

Fig. 1. SDS-PAGE of the identified protein from the hemolymph of *Mesocyclops leuckarti*.



S.A: *Staphylococcus aureus;* K.P: *Klebsiella pneumonia;* E.C: *Escherichia coli;* S.F: *Shigella flexneri*; S1: Hemolymph of plankton cultured without CUD; CUD: Cow urine distillate

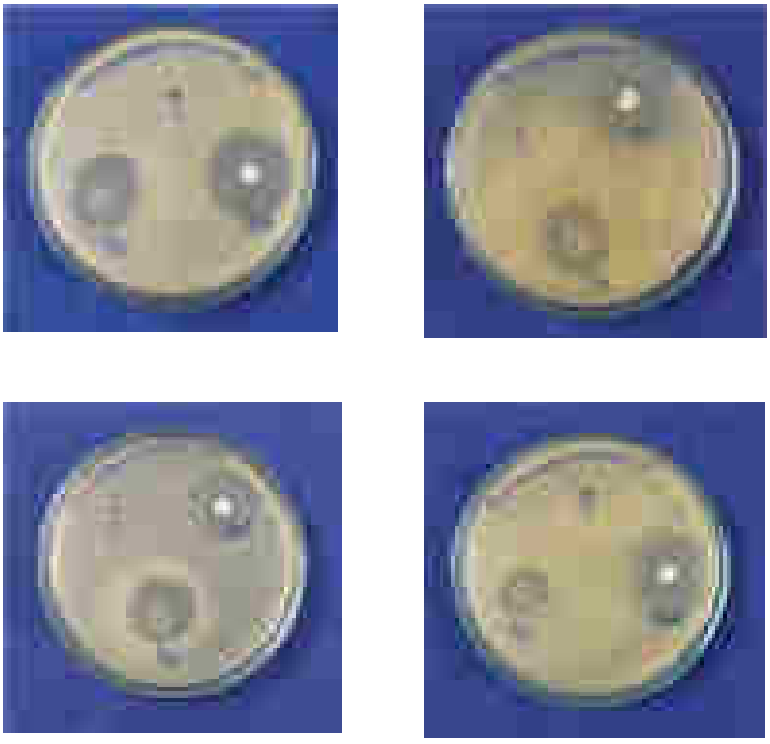
Fig. 3. Antibacterial activity high molecular protein of hemolymph of *Mesocyclops leuckarti* cultured without cow urine distillate.

leuckarti cultured without CUD showed antibacterial activity against *Klebsiella pneumonia* (14 mm) and no activity against other pathogens. However, the *M. leuckarti* organisms cultured in the presence of CUD possessed low molecular weight protein fractions that expressed antibacterial activity against all pathogens with maximum activity against *Klebsiella pneumonia* (18 mm) and minimum activity against *Shigella flexneri* (12 mm) as similar to the activity of high molecular weight protein fraction.

4. Discussion

A number of multidrug resistant bacterial species are listed by WHO that requires the urgent development of novel antimicrobials McKenna (2014). For the first time to the best of our knowledge, an attempt utilizing the antimicrobial proteins derived from the hemolymph of freshwater zooplankton *M. leuckarti* as a substitute for conventional antibiotics for the control of multidrug resistant bacterial isolates. The peptides derived from the hemolymph of invertebrates exhibit a broad spectrum of activities Mona Hajirasouli and Jamileh Pazooki, (2014) and these peptides mainly target the bacterial membrane. With the advent of artificial intelligence and bioinformatics tools unprecedented sequences and sequence combinatorial space of antimicrobial peptides have been generated (Porto, 2018a; Porto et al., 2018b). The present study documented the antimicrobial property of the hemolymph proteins from fresh water zooplankton against selected microbial pathogens. From the results it is evident that the proteins from the hemolymph were effective towards Gram- negative bacterial species compared to Gram positive bacteria. In agreement with our study, the antimicrobial proteins of *Charybdis lucifera* hemolymph exhibited effective antimicrobial activity against the Gram- negative bacteria *E.coli* (11 mm) and *P.aeruginosa* (11 mm) compared to the Gram positive bacteria Rameshkumar et al. (2009).

Several previous literatures reported the positive role of CUD in the treatment of cardiac, respiratory, kidney diseases (Ojewole and Olusi, 1976; Chauhan et al. 2001) and antimicrobial activity Yadav et al. (2008). The present study observed an increase in antibacterial activity of the hemolymph proteins against the selected human pathogenic bacterial species when cultured in the presence of CUD. This enriched antibacterial activity might be due to the fact that CUD itself possesses antibacterial, antifungal and antimicrobial activities (Hu et al. 2007; Shaw et al. 2007) Hence due to these properties, the plankton cultured in CUD media reflected the same properties by imbibing the active ingredients from CUD into hemocoel and hemolymph Badadani et al. (2007). A similar study on the antimicrobial activity of CUD against periodontal pathogens was also reported Gupta et al. (2017) and Bardvalli et al. (2016). Hemolymph of *Maydelliathelphusa masoniana* showed effective antibacterial activity against *Escherichia coli, Streptococcus pyrogenes, Pseudomonas aeruginosa, Staphylococcus aureus, Bacillus subtilis* and *Salmonella paratyphi* Rakesh et al. (2019). These results indicate that hemolymph have developed a variety of defense molecules against pathogenic microbes, but the degree of antimicrobial activity may vary depending upon the species Ojewole and Olusi (1976). It was reported in anearlier study that the concentration of hemolymph proteins exhibits wide inter specific variations and hemocytes might be the site of production and storage of these antimicrobial peptides. In the present study high molecular weight proteins of hemolymph exhibited effective antimicrobial property against the selective microbial pathogens



S.A: *Staphylococcus aureus;* K.P: *Klebsiella pneumonia;* E.C: *Escherichia coli;* S.F: *Shigella flexneri*; S2: Hemolymph of plankton cultured with CUD; CUD: Cow urine distillate

Fig. 4. Antibacterial activity high molecular protein of hemolymph of *Mesocyclops leuckarti* cultured with cow urine distillate.

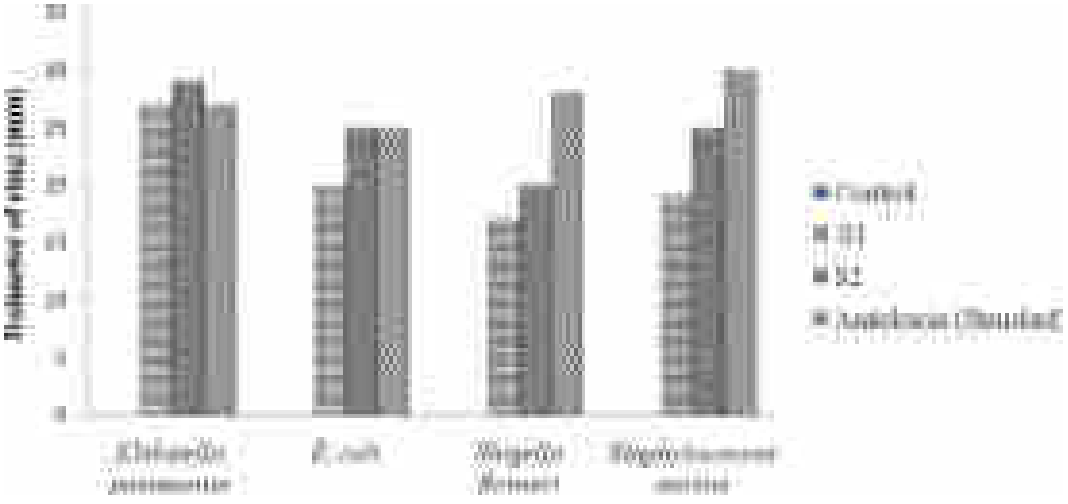
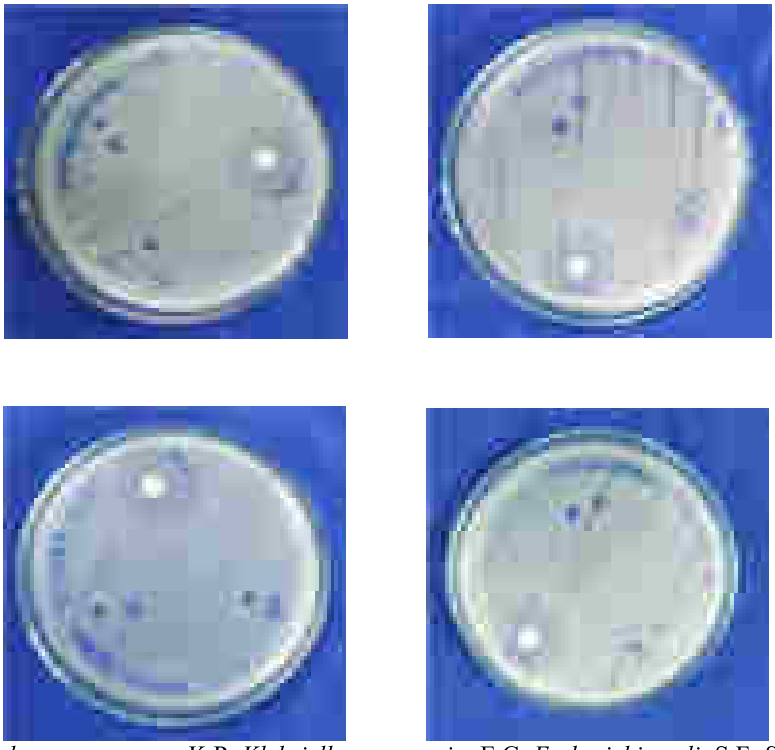


Fig. 5. Antibacterial activity of high molecular protein in hemolymph of *Mesocyclops leuckarti.*

compared to low molecular weight proteins of hemolymph. The supplement of CUD in the growth media further enhanced the antagonistic activity of the hemolymph proteins against microbial pathogens. Thus the present investigation indicates that the hemolymph proteins of *M. leuckarti* may contain potential antibiotics. The antimicrobial assay performed in this study may form a baseline information for further studies revealing the fact that *M. leuckarti* will provide an opportunity for the production of new natural alternatives for antibiotics

In conclusion, the present study reported the isolation of hemolymph proteins from the fresh water zooplankton *M. leuckarti* and evaluated its antagonistic activity against human pathogenic bacteria. Hemolymph proteins can be used as an alternative to conventional antibiotics since there is a global concern on the antibiotics overuse or misuse. Several issues have to be dwelt before the application of hemolymph proteins as antimicrobials such as production cost, in vivo efficacy, frequency of application and dosage etc. Overall, this study might be a promising solution



S.A: *Staphylococcus aureus;* K.P: *Klebsiella pneumonia;* E.C: *Escherichia coli;* S.F: *Shigella flexneri*; S1: Hemolymph of plankton cultured without CUD; CUD: Cow urine distillate

Fig. 6. Antibacterial activity low molecular protein of hemolymph of *Mesocyclops leuckarti* cultured without cow urine distillate.

S.A: *Staphylococcus aureus;* K.P: *Klebsiella pneumonia;* E.C: *Escherichia coli;* S.F: *Shigella flexneri*; S2: Hemolymph of plankton cultured with CUD; CUD: Cow urine distillate

Fig. 7. Antibacterial activity low molecular protein of hemolymph of *Mesocyclops leuckarti* cultured with cow urine distillate.

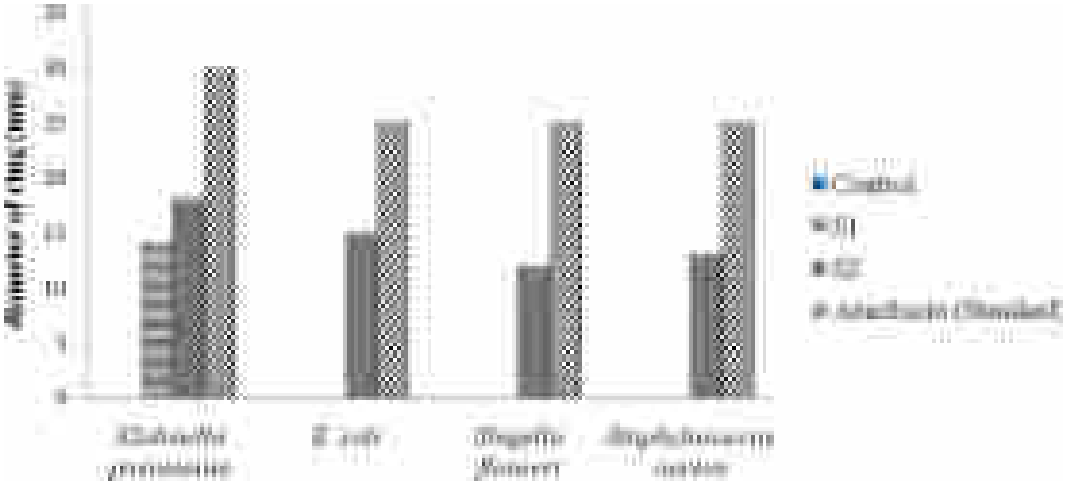


Fig. 8. Antibacterial activity of low molecular protein in hemolymph.

Table 2
Antibacterial activity of low molecular protein in freshwater zooplankton *Mesocyclops leuckarti* hemolymph against bacterial pathogens.

Samples	Zone of Inhibition in diameter (mm)			
	Klebsiella pneumonia	*Escherichia coli*	*Shigella flexneri*	*Staphylococcus aureus*
Control	–	–	–	–
S1	14mm	–	–	–
S2	18mm	15mm	12mm	13mm
Amikacin (Standard)	30mm	25mm	25mm	25mm

- = No zone
Control = Distilled water
S1 = Hemolymph of plankton cultured without cow urine distillate
S2 = Hemolymph of plankton cultured with cow urine distillate

for the production of alternative antimicrobial agents against drug resistant bacterial strains.

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