

**ECOLOGY, EXPERIMENTAL BIOLOGY AND ECO-TOXICITY
OF DIETARY MICROPLASTICS ON PHYSIOLOGICAL AND
BIOCHEMICAL RESPONSES OF MARINE COPEPOD
OITHONA DISSIMILIS (LINDBERG, 1940)**

**A Thesis submitted to
BHARATHIDASAN UNIVERSITY, TIRUCHIRAPPALLI
for the award of the degree of
DOCTOR OF PHILOSOPHY
IN
MARINE SCIENCE**

**By
Mr. P. RAJU**

***Under the guidance of*
Dr. P. SANTHANAM
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CERTIFICATE

This is to certify that the thesis entitled “**ECOLOGY, EXPERIMENTAL BIOLOGY AND ECO-TOXICITY OF DIETARY MICROPLASTICS ON PHYSIOLOGICAL AND BIOCHEMICAL RESPONSES OF MARINE COPEPOD *OITHONA DISSIMILIS* (LINDBERG, 1940)**” submitted by **Mr. P. RAJU** for the award of the Degree of Doctor of Philosophy in Marine Science is based on a result of studies carried out by him under my supervision and guidance at Department of Marine Science, School of Marine Sciences, Bharathidasan University, Tiruchirapalli-24, Tamil Nadu, India. This thesis or any part of this thesis has not been submitted elsewhere for the award of any other degree or diploma of this or any other University.

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DECLARATION

The research work presented in this dissertation entitled “**ECOLOGY, EXPERIMENTAL BIOLOGY AND ECO-TOXICITY OF DIETARY MICROPLASTICS ON PHYSIOLOGICAL AND BIOCHEMICAL RESPONSES OF MARINE COPEPOD *OITHONA DISSIMILIS* (LINDBERG, 1940)**” has been carried out under the guidance of **Dr. P. SANTHANAM**, Associate Professor, Department of Marine Science, School of Marine Sciences, Bharathidasan University, Tiruchirappalli-24. 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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W	URL: https://www.x-mol.com/paperRedirect/1475627649139777536 Fetched: 2022-03-28T13:37:16.9570000		18



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CONTENT

CHAPTER NO.	TITLE	PAGE NO.
Chapter I	1. GENERAL INTRODUCTION	1 - 7
	2. REVIEW OF LITERATURE	8 - 21
	3. SCOPE AND OBJECTIVES	22
	4. DESCRIPTION OF THE STUDY AREA	23 - 25
Chapter II	5. ECOLOGY OF MARINE COPEPODS FROM NAGAPATTINAM COASTAL WATERS	26 - 60
	5.1 Introduction	26
	5.2 Materials and Methods	27
	5.3 Results	31
	5.4 Discussion	53
Chapter III	6. MORPHOLOGICAL AND MOLECULAR IDENTIFICATION OF MARINE COPEPOD <i>OITHONA DISSIMILIS</i>	61 - 75
	6.1 Introduction	61
	6.2 Materials and Methods	62
	6.3 Results	67
	6.4 Discussion	73
Chapter IV	7. OPTIMIZATION OF CULTURE CONDITIONS FOR MARINE COPEPOD <i>OITHONA DISSIMILIS</i>	76 - 104
	7.1 Introduction	76
	7.2 Materials and Methods	77
	7.3 Results	83
	7.4 Discussion	101

Chapter V	8. IMPACT OF MICROPLASTICS INGESTION ON PHYSIOLOGY OF MARINE COPEPOD <i>OITHONA DISSIMILIS</i>	105 - 120
	8.1 Introduction	105
	8.2 Materials and Methods	107
	8.3 Results	109
	8.4 Discussion	118
Chapter VI	9. IMPACT OF MICROPLASTICS INGESTION ON NUTRITIONAL PROFILE OF MARINE COPEPOD <i>OITHONA DISSIMILIS</i>	121 - 134
	9.1 Introduction	121
	9.2 Materials and Methods	123
	9.3 Results	127
	9.4 Discussion	130
	10. SUMMARY AND CONCLUSION	135 - 137
	REFERENCES	138 - 178

LIST OF FIGURES

S. No.	TITLE	PAGE NO.
Fig. 1.	Map showing the sampling areas in Nagapattinam coastal waters	23
Fig. 2.	Seasonal variation of rainfall in Nagapattinam coastal waters	32
Fig. 3.	Seasonal variation of atmospheric temperature in Nagapattinam coastal waters	32
Fig. 4.	Seasonal variation of surface water temperature in Nagapattinam coastal waters	33
Fig. 5.	Seasonal variation of salinity in Nagapattinam coastal waters	33
Fig. 6.	Seasonal variation of pH in Nagapattinam coastal waters	34
Fig. 7.	Seasonal variation of dissolved oxygen in Nagapattinam coastal waters	34
Fig. 8.	Seasonal variation of Nitrate in Nagapattinam coastal waters	36
Fig. 9.	Seasonal variation of Nitrite in Nagapattinam coastal waters	37
Fig. 10.	Seasonal variation of Phosphate in Nagapattinam coastal waters	37
Fig. 11.	Seasonal variation of Silicate in Nagapattinam coastal waters	38
Fig. 12.	Seasonal variation of Ammonia in Nagapattinam coastal waters	38
Fig. 13.	Seasonal variations of chlorophyll 'a' in Nagapattinam coastal waters during February 2019 to January 2020	39
Fig. 14.	Seasonal variations of copepods population density in Nagapattinam coastal waters during February 2019 to January 2020	40
Fig. 15.	Seasonal variations of species diversity in Nagapattinam coastal waters during February 2019 to January 2020	41
Fig. 16.	Seasonal variations of species richness of copepods in Nagapattinam coastal waters during February 2019 to January 2020	42
Fig. 17.	Seasonal variations of species evenness in Nagapattinam coastal waters during February 2019 to January 2020	43

Fig. 18.	Seasonal variations of components plot in rotated space in Nagapattinam coastal waters during February 2019 to January 2020	44
Fig. 19.	Inverted phase-contrast microscopic (a) and scanning electron microscopic (b) images of <i>O. dissimilis</i>	67
Fig. 20.	Construction of inter-specific phylogenetic tree of COI gene of <i>O. dissimilis</i> PS-11 from its closely related sequences obtained from MEGA 7.0. Green color bullet differentiates our target sequences.	70
Fig. 21.	Construction of inter-specific protein based phylogenetic tree of COI gene of <i>O. dissimilis</i> PS-11 from its closely related sequences obtained from MEGA 7.0. Green color bullet differentiates our target sequences.	71
Fig. 22	Survival rate of <i>O. dissimilis</i> in respect to temperature	85
Fig. 23	Nauplii production rate of <i>O. dissimilis</i> in respect to temperature	85
Fig. 24	Population density of <i>O. dissimilis</i> in respect to temperature	86
Fig. 25	Survival rate of <i>O. dissimilis</i> with reference to salinity	88
Fig. 26	Nauplii production of <i>O. dissimilis</i> with reference to salinity	88
Fig. 27	Population density of <i>O. dissimilis</i> with reference to salinity	89
Fig. 28	Survival rate of <i>O. dissimilis</i> in respect to pH	91
Fig. 29	Nauplii production rate of <i>O. dissimilis</i> in respect to pH	91
Fig. 30	Population density of <i>O. dissimilis</i> in respect to pH	92
Fig. 31	Survival rate of <i>O. dissimilis</i> with reference to light intensity	94
Fig. 32	Nauplii production rate of <i>O. dissimilis</i> with reference to light intensity	94
Fig. 33	Population density of <i>O. dissimilis</i> with reference to light intensity	95
Fig. 34	Survival rate of <i>O. dissimilis</i> in respect to diets	97
Fig. 35	Nauplii production rate of <i>O. dissimilis</i> in respect to diets	97
Fig. 36	Population density of <i>O. dissimilis</i> in respect to diets	98
Fig. 37	Survival rate of <i>O. dissimilis</i> with reference to diet concentration	100

Fig. 38	Nauplii production rate of <i>O. dissimilis</i> with reference to diet concentration	100
Fig. 39	Population density of <i>O. dissimilis</i> with reference to diets concentration	101
Fig. 40	Effect of MPs ingestion on survival rate of <i>O. dissimilis</i>	110
Fig. 41	Effect of MPs ingestion on Nauplii production rate of copepod <i>O. dissimilis</i>	111
Fig. 42	Effect of MPs ingestion on population density <i>O. dissimilis</i>	112
Fig. 43	Effect of MPs ingestion on biochemical composition of copepod. <i>O. dissimilis</i>	128

LIST OF TABLES

S. No.	TITLE	PAGE NO.
Table 1.	Techniques used for the analysis of physico-chemical and biological characteristics of Nagapattinam coastal waters sample.	29
Table 2.	Correlation matrix among the physico-chemical and biological characteristics of Nagapattinam coastal waters during February 2019-January 2020 at station 1	45
Table 3.	Correlation matrix among the physico-chemical and biological characteristics of Nagapattinam coastal waters during February 2019-January 2020 at station 2.	46
Table 4.	Correlation matrix among the physico-chemical and biological characteristics of Nagapattinam coastal waters during February 2019-January 2020 station-3.	47
Table 5.	List of copepod species recorded in Nagapattinam coastal waters during February 2019- January 2020	48
Table 6.	R mode Varimax sorted factor analysis of physico-chemical and biological parameters of Nagapattinam coastal waters.	51
Table 7.	PCR reaction mix was used for molecular analysis.	64
Table 8.	The primers used for copepod molecular analysis.	65
Table 9.	The conditions were maintained in PCR program	65
Table 10.	Dataset preparation of cytochrome c oxidase I of <i>O. dissimilis</i> PS-11 and its phylogenetic similarity using NCBI-BLAST	70
Table 11.	Estimation of inter-specific pair-wise genetic distance of COI gene of <i>O. dissimilis</i> PS-11 from its phylogenetic neighbors' obtained from MEGA 7.0	71
Table 12.	Estimation of inter-specific pair-wise genetic distance of COI gene of <i>O. dissimilis</i> PS-11 from its phylogenetic neighbors' obtained from MEGA 7.0.	72
Table 13.	Effect of temperature on post embryonic development of marine copepod <i>O. dissimilis</i>	86
Table 14.	Effect of salinity on post embryonic development of marine copepod <i>O. dissimilis</i>	89

Table 15.	Effect of pH on post embryonic development of marine copepod <i>Oithona dissimilis</i>	92
Table 16.	Effect of light intensity on post embryonic development of marine copepod <i>O. dissimilis</i>	95
Table 17.	Effect of diets on post embryonic development of marine copepod <i>O. dissimilis</i>	98
Table 18.	Effect of diets concentration on post embryonic development of marine copepod <i>O. dissimilis</i>	101
Table 19.	Effect of MPs ingestion on post embryonic development of marine copepod <i>O. dissimilis</i>	113
Table 20.	Effect of MPs ingestion on amino acids profile of <i>O. dissimilis</i>	129
Table 21.	Effect of MPs ingestion on fatty acids profile of copepod <i>O. dissimilis</i>	130

LIST OF PLATES

S. No.	TITLE	PAGE NO.
Plate 1.	Station-1 (Neritic Zone)	25
Plate 2.	Station-2 (Sea Mouth)	25
Plate 3.	Station-3 (Kaduvaayar estuary)	25
Plate 4.	Collection of water and copepod samples from Nagapattinam coastal waters	30
Plate 5.	Microscopic images of some calanoid copepods recorded in Nagapattinam coastal waters during February 2019 -January 2020	49
Plate 6.	Microscopic images of some cyclopoid and harpacticoid copepods recorded in Nagapattinam coastal waters during February 2019 - January 2020	50
Plate 7	Microalgae culture facility	78
Plate 8	Copepod culture facility	79
Plate 9	Experimental setup for the survival of copepod <i>O. dissimilis</i>	80
Plate 10	Experimental setup for the nauplii production rate of copepod <i>O. dissimilis</i>	81
Plate 11	Experimental setup for population density of copepod <i>O. dissimilis</i>	82
Plate 12	Microscopic observation on post embryonic development of <i>O. dissimilis</i>	83
Plate 13	Post-embryonic developmental stages of copepod <i>O. dissimilis</i>	104
Plate 14	The accumulation of MPs (2.0 μ m) in the gut of <i>O. dissimilis</i>	114
Plate 15.	The accumulation of MPs (2.0 μ m) in the antenna of <i>O. dissimilis</i>	114
Plate 16	The accumulation of MPs (2.0 μ m) in the leg region of <i>O. dissimilis</i>	115

Plate 17	The accumulation of MPs (2.0 μm) in the urosome region of <i>O. dissimilis</i>	115
Plate 18	The accumulation of MPs (0.5 μm) in the gut region of <i>O. dissimilis</i>	116
Plate 19	The accumulation of MPs (0.5 μm) in the gut and urosome region of <i>O. dissimilis</i>	116
Plate 20.	The accumulation of MPs (0.1 μm) in the gut of <i>O. dissimilis</i>	117
Plate 21.	The accumulation of MPs (0.1 μm) in the egg sac of <i>O. dissimilis</i>	117

LIST OF ABBREVIATIONS

%	Percentage
±	Plus or minus
μl	Microliter
μm	Micrometer
PSU	Practical salinity unit
°C	Degree Celsius
CM	Centimeter
‰	Parts per thousand
Fig	Figures
mg	Milligram
ml	Milliliter
mm	Millimeter
N	Nauplii
C	Copepodite
pH	Hydrogen-ion Concentration
sp.	Species
PO ₄ ³	Phosphate
NO ₃	Nitrate
NO ₂	Nitrite
NH ₃	Ammonia
SiO ₃	Silicate
PCA	Principle Component Analysis
ISO	<i>Isocrysis galbana</i>
CHL	<i>Chloroella marina</i>
PICO	<i>Picochlorum maculatum</i>

NAN	<i>Nannochloropsis oculata</i>
AMS	<i>Amphora subtropica</i>
SR	Survival rate
NPR	Nauplii production rate
PD	Population density
PEM	Post embryonic development
FRP	Fiber Reinforced Products
NCBI	National Centre for Biotechnology Information
DT	Development time
GT	Generation time
GC-MS	Gas chromatography-Mass Spectrum
NEAA	Non essential amino acid
EAA	Essential amino acids
OD	Optical density
TD	Thermal desorption
TLC	Thin layer chromatography
UV	Ultraviolet
HPTLC	High-performance thin-layer chromatography
EPA	Eicosapentaenoic Acid
DHA	Docosahexaenoic acid
PP	Polypropylene
PS	Polystyrene
PA	Polymeric amide
PES	polyester
PVS	Polyvinyl chloride
HDPE	High-Density polyethylene
LDPE	Low-Density Polyethylene
PP	Polypropylene

SFA	Saturated fatty acids
PUFA	Poly unsaturated fatty acids
MUFA	Monounsaturated fatty acids
g	Gram
mg	Milligram
Ind.	Individual
MFS	Millipore filtering system
ST	Station
SD	Species Diversity
SR	Species Richness
SE	Species Evenness
AT	Atmospheric temperature
SWT	Surface water temperature
PCR	Polymerase Chain Reaction
DNA	Deoxyribonucleic acid
rRNA	Ribosomal ribonucleic acid
COI	Cytochrome c oxidase subunit I
NJ	Neighbor joining
A1	Antenna

Chapter I

1. Introduction

Copepods

Marine ecosystem is the largest and the most diverse ecosystem which covers 71% of the water in the planet. The marine ecosystems are defined by their unique biotic (living) and abiotic (nonliving) factors. Biotic factors includes plants, animals, and microbes and abiotic factors includes sunlight, amount of oxygen and nutrients dissolved in the water, depth, and temperature. Copepods are in the form of free-living, symbiotic, internal or external parasites found almost in every major metazoan phylum. They live in a wide range of salinity, from fresh to hypersaline, and can be found almost everywhere. And even from subterranean caves to pools collected in bromeliad leaves or damp leaf litter on the ground, from streams, rivers, and lakes to the open ocean and the sediment layers beneath. Their habitats range from the deepest ocean trenches to highest mountain lakes, as well as the cold even in polar ice-water interface and hot active hydrothermal vents. The word copepod originates from the Greek words Kope ("oar") and Podos ("foot"), referring to how their antennae and legs move like oars as the copepod is swimming. Copepods are small crustacean found in almost all aquatic environments. They are most abundant in the marine habitat, and many of the species also occupies the freshwater or estuarine environment as well. The orders of copepods that are most interesting for aquaculture are the Calanoida, Harpacticoida and Cyclopoida. The planktonic group is divided into two major divisions called phytoplankton and zooplankton whereas the phytoplankton are the primary producers and minute photosynthetic plants and zooplankton are the secondary producers and primary consumers of the marine food web. Among the zooplankton, the copepods are the largest and most diversified group which comprises over 80% of the total zooplankton population

and one of the most abundant animals on the planet. It is estimated that there are 24,000 species in copepods comprises of 2400 genera, 210 families (<http://www.luciopesce.net/copepods/intro.htm>). The body length, size of adult's ranges between 1 and 2 mm. Adults of free-living species may be as short as 0.2 mm or as long as 17 mm. In the case of parasitic forms on large vertebrate hosts, body lengths may exceed 20 cm. Ecologically, the planktonic copepods provide functionally important links in the aquatic food chain, feeding on the microscopic algal cells of the phytoplankton and, in turn, being eaten by juvenile fish and other planktivores, including some whales. In fresh water, copepods have the potential to act as a biological control mechanism for malaria by consuming mosquito larvae. However, they also serve as intermediate hosts of many animal parasites and even parasites of humans, including the fish tapeworm and guineaworm. The copepods are the dominant forms of the marine plankton and constitute the secondary producers in the marine environments and a fundamental step in the trophodynamics of the oceans. They are found in abundance and constitute an important source of protein in the oceans. Copepods represent an important food source for a number of fish and shrimp species, most copepod species live in the benthic zone. Copepods are widely known to be a highly important source of live food for fish larvae rearing. Despite major advancements in copepod cultivation, its usage remains rare. This is due to the fact that copepods are rarely used in commercial settings. As a result, significant efforts must be taken to scale up copepod culture to commercial levels in order to assure the generation of a consistent, large-scale supply of copepods. Copepods come in a variety of shapes, sizes, and movements, and we may learn about them by looking at them (Perumal *et al.*, 2015).

According to the statement of Stottrup (2006) copepods are numerous, the number of species cultivated for aquaculture is limited to three orders, which include (i.e) Cyclopoida, Harpacticoida and Calanoida (Støttrup, 2006). For Cyclopoida around half of there are

marine and rest of them are in inhabits freshwater environments (Mauchline, 1998). Cyclopoida are both commersal, parasitic and pelagic associated (Mauchline, 1998) in nature. A common trait for cyclopoida is that they are egg carriers (Støttrup, 2006). Cultures of Cyclopoida are the least studied organisms of the three orders and the genera *Oithona* spp., *Paracyclops* spp. and *Apocyclops* spp. seems to be the best candidates for live feed for marine fish larvae (Lee *et al*, 2013; Støttrup, 2006). Harpacticoida are primarily marine species (~90%) (Mauchline, 1998). They are mainly epibenthic associated, egg carriers, and the best candidates for aquaculture appear to be the genera *Euterpina* spp., *Tigriopus* spp., and *Tisbe* spp. (Støttrup 2006). Around 75% of the Calanoida are marine, and they are mainly pelagic (Mauchline, 1998). Most calanoids are broadcasters, shedding their eggs individually directly in the water column (Støttrup, 2006). Calanoid copepods are the most studied copepod order, containing the most studied genera in the world, *Acartia* spp. is closely followed by *Calanus* spp., together with *Temora* spp., *Centropages* spp., *Pseudocalanus* spp. and *Paracalanus* spp. (Støttrup, 2006).

Several researchers suggested many strong reasons that cyclopoida marine copepod can be cultured at mass scale level for marine fish larvae, the reasons are: 1. They are raised at high densities in small volumes, 2. Planktonic different stages for nauplii (6), copepodite (5) and adult (1), 3. They have a short generation time, 4. High level reproductive capacity, 5. The ability to utilize different food source such as live foods, inert diets, vegetables and manure. 6. Tolerate a wide range of environmental conditions like temperature, salinity, pH, light intensity etc. 7. Capable of fertilizing up to 12 to 21 broods from single fertilization, 8. To synthesis significant amount of EPA and DHA (Støttrup, 2000; Santos *et al.*, 2006; Drillet, 2010; Ajiboye *et al.*, 2011; Santhanam and Perumal 2011; Ananth and Santhanam, 20011; Jeyaraj *et al.*, 2014; Kaviyarasan *et al.*, 2019)

Microplastics

Plastic pollution is one of the most pressing environmental issues and social concerns of the 21st century. The unique characterization of plastic, for instance, flexibility, durability, and easy portability make them attractive for continuous usage in all our day-to-day life activities. Global plastics production was estimated to be 367 million metric tons in 2020 (Tiseo, 2022). Plastic waste is being generated at an exponential rate, which has led to a significant increase of plastic debris in marine ecosystems over recent decades (Ostle *et al.*, 2019). The foremost commonly used plastics are polypropylene (PP), polystyrene (PS), polyamide (PA), polyester (PES), polyvinylchloride (PVC), high-density polyethylene (HDPE), low-density polyethylene (LDPE), and polyethylene terephthalate (Alimba *et al.*, 2019). Improper management of these plastic has resulted in the significant increase of microplastics in the environment due to its degradation or fragmentation.

Any synthetic polymeric matrix with a size range of 1µm to 5µm, of either regular or irregularly shaped materials can be defined as Microplastics (Frias and Nash, 2019). In terms of the origin, Microplastics are of two types namely, primary and secondary microplastics. Primary microplastics are microplastics which are manufactured in micron sizes for commercial usage. Secondary microplastics are the degraded or fragmented larger plastic materials. Both the primary microplastics and secondary microplastics are found in the environment globally (Wang *et al.*, 2021; Li *et al.*, 2016).

Every year, influnce of plastics entering into the ocean was estimated to be 4 to 12 million tonnes (Jambeck. 2015). Anthropogenic activities like domestic, industrial and coastal activities are the major sources of plastic pollution in the marine environment. Besides littering and mismanaged wastes, primary microplastics are becoming major source of concern. Their release in the aquatic environment is much less visible, resulting from the deliberate addition of microbeads in cosmetics products or from the abrasion of textiles or

tyres (Boucher *et al.*, 2017). Disintegration of common polymers in the environment is considered as the source of secondary microplastic pollution. This is achieved by UV-exposure, photo-oxidative degradation, microbial degradation, chemical or mechanical weathering (Browne *et al.*, 2013). Microplastics (MPs) prevalence within the environment has been documented in virtually within almost every crevices on the earth, from open to confined ocean environment, from surface water to sea sediments, from the equator to the polar regions, from drinking water to waste water and even present in the human placenta (Ragusa *et al.*, 2021; Peng *et al.*, 2017). MPs are consistently detected in aquatic environments, including freshwater lakes, rivers, beaches, ocean waters, deep-sea sediments, and tributaries primarily caused by poor waste management (Ballent *et al.*, 2016). Sea-based sources for plastics include majorly fishing, shipping and transportation, whereas land-based sources include tourism, industrial and riverine inputs to the coast, seas and oceans (Browne *et al.*, 2010).

After entering into the aquatic environment, MPs can be distributed in different water layers like surface water, water column and bottom sediment based on the property (density, shape and polarity) of polymer and water flow conditions. This distribution influence their bioavailability and toxicity to the aquatic biota (Wang *et al.*, 2019). Generally, MPs which are lower in density (Eg. polystyrene, polypropylene and polyster) tends to present in the water column and high-density MPs (Eg. Polyvinylchloride, Poly amide) tends to settle down in the bottom. But storms, riverine input and biofouling of microplastics can lead to vertical distribution in the aquatic environment (Browne *et al.*, 2010). Microplastics are promptly bioavailable to a range of aquatic organisms by virtue of their minuteness (<5mm). In the aquatic environment, microplastics are not directly ingested by different organisms intentionally or unintentionally, but also indirectly transferred from low to high trophic levels via aquatic food chains (Wang *et al.*, 2019). These MPs cause

detrimental effect to the biological organisms. Ingestion of microplastic particles has been recorded in a most of the species, including; zooplankton, fish, seabirds, decapod crustaceans, mussels, amphipods, lugworms and barnacles. However, it is considered that it is those species at lower trophic levels that are most susceptible to microplastic ingestion (Huang *et al.*, 2020; Wright *et al.*, 2013). Phytoplankton are the vital primary producer at the bottom of the aquatic food chain and are essential in global oxygen production, in addition to the nitrogen and phosphorus biogeochemical cycle (Cherchi *et al.*, 2011). Larger microplastics can lead to cause adverse effects in microalgae by blocking the light and influencing photosynthesis, while smaller microplastic destroys the algae cell wall by attaching to the phytoplankton surface (Liu *et al.*, 2020b). Zooplankton is group of drifting heterotrophs, which play a vital role in the transportation of energy along with that they also passes the pollutants to successive community (Lee *et al.*, 2013). These zooplankton especially filter feeders likely to ingest the MPs automatically while they are feeding. MPs interact with the zooplankton like copepod, rotifer and cladocera, by altering the feeding behaviour or by surface adherence. As in the case of the primary predator, notably, exposure of microplastics result in significant size-dependent effects on zooplankton, such as feeding capacity, increased mortality, long reproduction time, reduced growth rate and fecundity, and even affect the next generation (Besseling *et al.*, 2014, Lee *et al.*, 2013).

In General, the smaller plastic particles including micro and nanoplastics are more toxic and harmful to zooplankton (Lee *et al.*, 2013, Rist *et al.*, 2017). Moreover, the excretion ability of microplastics may be significantly correlated with its particle size. Polystyrene (PS), a thermoplastic polymer characterized by highly transparent, durable and can be easily dyed and used for the production of CDs, toys, toothbrushes and styrofoam. Styrofoam is widely used in manufacturing some food containers like trays, plates and cups and for packaging materials (Kik *et al.*, 2020). According to the report from merchant

research and consulting shows that around 32.7 million tonnes of polystyrene was produced worldwide (Farrelly and Shaw, 2017). Polystyrene is considered as a model microplastics in majority of the studies focusing on the effect of characteristic particle surfaces on various biological parameters, due to their easy synthesizing nature over a broad range of sizes (Loss *et al.*, 2014). However, the knowledge on the toxicity and effect of polystyrene microplastics on the aquatic marine invertebrate are inconsiderable and very scarce in India.

Thus, the present investigation focused on the diverse aspects viz; 1) Ecology of copepod from Nagapattinam coastal waters 2) Morphological and Molecular Identification of copepod *Oithona dissimilis* 3) Optimization of culture conditions for copepod *Oithona dissimilis* 4) Impact of microplastics ingestion on physiology of marine copepod *Oithona dissimilis* and 5) Impact of microplastics ingestion on biochemical profile of copepod *Oithona dissimilis*

2. Review of Literature

Ecology of Copepods

To our knowledge, the first recorded work on systematics of copepod was studied by Davis (1949), who described the seasonal abundance and distribution of copepods in the waters of the Northeastern Pacific Ocean; Uye (1997) from the Japan Sea; Licandro and Ibanez (2000) from the Gulf of Trigullio (Ligurian Sea, Western Mediterranean); Bamstedt, (2000) from the West coast of Swedish; Olympia *et al.* (2000) from the Northern Adriatic Sea; Osore *et al.* (2003) from Makupa creek and Mombasa Harbour in Kenya; James Mwaluma *et al.* (2003) from Mida Creek, Kenya; Rezai *et al.* (2004) from Straits of Malacca Malaysia; Marcus (2004) from Coastal Zone of USA; Tan *et al.* (2004) from estuary, China; Lo *et al.* (2004) from Tapong Bay, Southwestern Taiwan; Frisch *et al.* (2006) from Donana, South-West Spain; Sterza and Fernande (2006) from Vitória Bay estuarine system (Southeastern Brazil); Magalhaes *et al.* (2006) observed the spatial and temporal distribution in density and biomass of two *Pseudodiaptomus* sp. in the river estuary; Belmonte (2006) studied the copepod fauna in small ponds of the Pollino National Park (South Italy), on seasonality and biometry of species; Hsu *et al.* (2008) from Lagoon in Taiwan; Dias *et al.* (2009) from tropical estuarine region in Brazil; Primo *et al.* (2009) from southern temperate estuary, Portugal; Chew and Chong (2011) from Sangga estuary in Malaysia; Telesh *et al.* (2011) from Baltic Sea; Kosobokova *et al.* (2011) from Arctic's central basins; Hui Hsiao *et al.* (2011) from East China Sea.

Shin-ichi Saitoh *et al.* (2011) studied the species diversity and community structure of pelagic copepods. Katerina Sevastou *et al.* (2011) studied the diversity of harpacticoid copepods in two Mediterranean micro-tidal sandy beaches. Zervoudaki *et al.* (2011) reported the copepod communities, production, and grazing in the Turkish Straits System and the

adjacent northern Aegean Sea during spring. Brugnano *et al.* (2012) studied the spring diel vertical distribution of copepod abundances and diversity in the open Central Tyrrhenian Sea (Western Mediterranean). Kohei Matsuno *et al.* (2012) studied the horizontal distribution of calanoid copepods in the Western Arctic Ocean. Chaalali *et al.* (2013) analysed the changes in the distribution of copepods in the Gironde estuary. Pepin *et al.* (2013) observed the distribution and feeding pattern of *Benthosema glaciale* in the western Labrador Sea was recorded. In which Fish-zooplankton interaction and the consequence to calanoid copepod populations. Gubanova *et al.* (2014) studied the species composition of Black Sea marine planktonic copepods. Yamaguchi *et al.* (2014) assessed the seasonal changes on zooplankton abundance, biomass, size structure, and dominant copepods were analyzed using an optical plankton counter. Hwang *et al.* (2014) carried out the copepod assemblage in the Northern South China Sea. Olivier *et al.* (2014) recorded out the seasonal fluctuation of the copepod resting egg bank in the middle Seine estuary in France.

In India, the studies on the distribution and abundance of zooplankton are widely based on the reports of Sewell (1912, 1932, 1940, 1948, and 1949). Krishnaswamy (1951a, 1952 a, 1953a, b, c, and 1956) has recorded valuable information on the systematics of copepods in the inshore waters of Madras. Seshadri (1957) determined the seasonal variation of the zooplankton for the Parangipettai coast. Several accounts with respect to the east coast of India are available on copepod distribution. Ummerkutty (1967a) from the Gulf of Mannar; Chandramohan (1977) from the Godavari estuary; Madhupratap *et al.* (1981) from the Andaman Sea; Bhunia and Choudhury (1982) from the Chemaguri creek of West Bengal; Gajbhiye *et al.* (1984) from the Versova creek of Mumbai; Baidy and Choudhury (1984) and Sarkar *et al.* (1986) from the Hooghly estuary; Murugan and Ayyakkannu (1991) from Uppanar backwaters; Maruthanayagam and Subramanian (1999) from the Palk Bay and Gulf of Mannar. Shanmugam *et al.* (1986), Godhantaraman (1994), Ramakrishnan *et*

al. (1999), Karuppasamy and Perumal (2000), Kathiresan (2000), Kathiresan and Bingham (2001) and Thiyagarajan *et al.* (2002) from the Pichavaram mangroves; Santhanam and Perumal (2003) and Perumal *et al.* (2008) from Parangipettai coastal waters. Vengadesh Perumal *et al.* (2009) studied the plankton diversity in the Kaduviyar estuary; Damotharan *et al.* (2010) from Kodiakkarai coastal waters; Shanthi and Ramanibai (2011) studied the copepods from Chennai Coast (Cooum and Adyar); Pillai *et al.* (2011) observed mesozooplankton distribution from the Andaman Sea. The community structure of harpacticoids from the Chennai coast was done by Mantha *et al.* (2012). Stephen *et al.* (2013) studied the zooplankton from Mumbai salt pans, and Ansari *et al.* (2013) analysed the community structure of harpacticoid copepods from the Southeast continental shelf of India. Santhanam *et al.* (2013) reported the copepod diversity from Parangipettai coastal ecosystem. Bozkurt and Can (2014) determined the seasonal variations in body length and fecundity of copepods *Thermocyclops crassus* and *Eudiaptomus drieschi* from Turkey (waters) Bode *et al.* (2014) reported the Spatio-Temporal variability of copepod abundance in the Northern Benguela upwelling system. Kitahashi *et al.* (2014) studied the diversity of harpacticoid copepods from the Ryukyu trench, Japan. Lodi *et al.* (2014) observed the patterns of the zooplankton population in a tropical reservoir. Bode *et al.* (2014) observed the Spatio-temporal variability of copepod abundance in the Northern Benguela upwelling system for 2005-2011. Kitahashi *et al.* (2014) examined the diversity of harpacticoid copepods from the Ryukyu trench, Japan. Lodi *et al.* (2014) observed the patterns of the zooplankton population in a tropical reservoir. Ananth (2015) inspected the diversity of copepod in Pambanar estuary, Southeast coast of India. Rajthilak *et al.* (2015) studied the distribution of copepods in Vellar estuary, Southeast coast of India. Rajthilak *et al.* (2016) analysed the spatial and temporal distributions of calanoid copepods (Crustacea; Arthropoda) along the Tamil Nadu coast, Southeast India. Santhanam *et al.* (2018) observed

the seasonal composition and diversity of zooplankton from the Muthupet mangrove wetland ecosystem, Southeast coast of India.

Experimental biology of copepods

The influence of temperature and food type on the growth and metabolism of some marine copepods was studied by Mullin and Brooks (1973). Breteler and Gonzalez (1982) have analysed the influence of food concentration on body length of calanoid copepods; Schnack *et al.* (1989) studied the variation in body length of cyclopoid copepods, *Oncaea venusta*, and *Oncaea media* from the Central Red Sea. Seasonal changes in the size of the calanoid copepods, *Pseudocalanus* accepts and *Acartia longicornis* were examined by Fredrika Norrbin (1993). Liang *et al.* (1996) observed the body length variations in *Centropages*. The morphometric and the length variations were determined in *Acartia* by Liang and Uye (1996 a) and seasonal variation in body length of *Paracalanus* sp. were observed by Liang and Uye (1996 b) from the Inland Sea of Japan. The size variations in different stages of *Pseudodiaptomus marinus* were determined by Liang and Uye (1997). Richardson and Verheye (1999) discussed the variations in body size of some calanoid copepods caused by food.

Bamstedt (2000) described the monthly variations in the prosome length of adult females and males of *Calanus finmarchicus*. Longoria *et al.* (2003) studied the effect of temperature and salinity in *Acartia* species; Knuckey *et al.* (2005) have experimented the development of an optimal microalgal diet for the culture of the *Acartia sinjiensis*. Effects of diets on the growth of the *Paracyclops nana* was analysed by Lee *et al.* (2006). Chen *et al.* (2006) have observed the reproduction and survival rate in *Pseudodiaptomus annandalei*; Milione and Zeng (2007) reported the effects of algal diets on population growth and egg hatching success of *Acartia sinjiensis* population growth and production of *Apocyclops dengizicus* fed on different diets was carried by Farhadian *et al.* (2008). Camus

and Zeng (2008) studied the effects of photoperiod on egg production and hatching success, in *Acartia sinjiensis*; Camus and Zeng (2009) studied the egg production and hatching success in *A. sinjiensis*; Cruz *et al.* (2009) and Rhyne *et al.* (2009) in *Pseudodiaptomus pelagicus*; Zaleha and Jamaludin (2010) in *Pararobertsonia* sp. Dhanker *et al.* (2012) studied the presentation of *Pseudodiaptomus annandalei* (Copepoda: Calanoida) on rotifer prey: Size selection, egg predation, and effect of algal diet. Matias-Peralta *et al.* (2012) observed the reproductive performance, growth, and development time of a tropical harpacticoid copepod, *Nitocra affinis* fed with different microalgal diets. Santhanam and Perumal (2012a, 2012b). Studied the effect of temperature, salinity, food concentration on the fecundity of *Oithona rigida*. Zang *et al.* (2013) studied the effects of different monoalgal diets on egg production, hatching success, and apoptosis induction in a Mediterranean population of the calanoid copepod *Acartia tonsa*. Santhanam *et al.* (2013) made an attempt on the effect of temperature and algal food on egg production and hatching of a calanoid copepod, *Paracalanus parvus*. Noyon and Froneman (2013) studied the variability in the egg production rates of the calanoid copepod, *Pseudodiaptomus hessei* in a South African estuary in relation to environmental factors. Effects of food concentration on egg production and feeding rates of the cyclopoid copepod *Oithona davisae* was carried out by Terol and Saiz (2013).

Bhuvaneshwari (2013) studied the effect of pH on feeding, survival, and egg production of copepod *Pseudodiaptomus annandalei*. The developmental biology of brackishwater copepod *Oithona rigida* was studied by Santhanam and Perumal (2013). Lee *et al.* (2014) studied the toxicity of dinoflagellate on marine copepod *Tigriopus* sp. Vu *et al.* (2014) examined the comprehensive and precise quantification of the calanoid copepod *Acartia tonsa* (Dana) for intensive live feed cultures using an automated Zoo Image system. Samba *et al.* (2014) examined the impact of the diatom-derived compounds on the feeding,

survivorship, and reproductive success of the calanoid copepod *Temora stylifera*. Effect of algal species and concentration on the development and fatty acid composition of two harpacticoid copepods, *Tisbe* sp. and *Tachidius discipes* was studied by Arndt and Sommer (2014). Noyon and Froneman (2014) reported the diet performance of the calanoid copepod, *Pseudodiaptomus hessei*, in a permanently open Southern African estuary inferred from fatty acid analyses. Isari *et al.* (2014) experimented on light-induced changes in the feeding behavior of the calanoid copepod *Clausocalanus furcatus*. Drillet *et al.* (2014) studied the production capabilities of *A. tonsa* when stocked at densities and tested the quality of the produced eggs. Nandakumar *et al.* (2014) studied the effects of temperature, feed, and salinity on the reproduction of *Macrosetella gracilis*. Anzueto-Sanchez *et al.* (2014) conducted a series of experiments on the effects of food concentration and temperature on the development, growth, reproduction, and survival of the copepod *Pseudodiaptomus euryhalinus*. Engstrom-Ost *et al.* (2014) conducted a laboratory study on the effects of short-term pH decrease on the reproductive output of the copepod *Acartia bifilosa*. Venkatesan (2014) studied the effect of pH on fecundity, survival, and feeding of copepod *Oithona rigida*.

Fereidouni *et al.* (2015) examined the effects of five photoperiod regimes on reproductive traits, development and generation time, survival rate, adult sex ratio, and total life span of cyclopoid copepod, *Mesocyclops* sp. Santhanam *et al.* (2015) observed and reported the effect of a monoalgal diet on the growth, survival, and egg production in *Nannocalanus minor*. Jayalakshmi *et al.* (2016a) studied the effect of acidification on fatty acids profiling of marine benthic harpacticoid copepod *Parastenhelia* sp. Jayalakshmi *et al.* (2016b) analysed the effect of temperature and salinity on survival and nauplii production rate of *Parastenhelia* sp. Nandakumar and Santhanam (2019) studied the feeding, survival, fecundity, and post embryonic development of zooplankton *Nitocra affinis*.

Culture of Copepods

Various researchers have attempted to mass culture the different species of copepods. Zillioux (1969) who is the first one to describe a continuous culture system for planktonic copepod; Corkett (1970) standardized the techniques for rearing the marine calanoid copepods. Kitajima (1973) opined that the harpacticoid copepod, *Tigriopus japonicus* is the most promising copepod for mass culture; Abraham and Gopalan (1975) and Gopalan (1977) have cultured the copepod *Nitocra spinipes*. Ikeda (1977) have stated that the small-sized neritic or brackishwater copepods are easy to rear artificially as compared with those of larger sized species; Omori and Ikeda (1984) gave an account on the zooplankton culture. Certain workers have successfully cultured the copepods in intensive outdoor systems; Hardy (1978); Paffenhofer (1979); R; Nellen (1981); Kahan *et al.* (1981); Uye (1982); Stottrup *et al.* (1998).

According to the Stamentment of Stottrup (2000), there are several culture techniques have been developed and practiced at the laboratory scale to provide small numbers of organisms needed for experimental work which focused on their nutrient, reproduction is physiological aspets. Concordantly, progress towards mass culture techniques of copepods has been slow and not thoroughly researched in larviculture. In addition, the development of this field has been fragmentary (Stottrup 2000). Payne and Ripplinge (2000) and Stottrup (2000) successfully cultured the copepod and used it in fish and shrimp hatcheries. Recently, increasing attention has been focused on copepod nauplii used as a potential high-quality live feed source for aquaculture (Stottrup, 2000; Payne *et al.*, 2001). The superiority of copepods for larviculture of marine fish has recently increased the interest for the controlled culture of copepods (Stottrup, 2003). McKinnon *et al.* (2003) reviewed the potential of tropical *Paracalanoid* sp. (*Bestiolina similis* and *Parvocalanus crassirostris*) as live feeds in aquaculture; Olivotto *et al.* (2008a) successfully cultured the

harpacticoid copepods, *Tisbe* spp. Olivotto *et al.* (2008b) successfully cultured the mediterranean calanoid copepod *Centropages typicus*; Guangxing and Donghui (2009) cultured calanoid copepod *Schmackeria poplesia* and Cassiano *et al.* (2011) cultured calanoid copepod *Pseudodiaptomous pelagicus*.

Ylenia Carotenuto *et al.* (2012) experimented on multigeneration cultivation of the copepod *Calanus helgolandicus* in a re-circulating system. Finiguerra *et al.* (2013) studied the effect of sex-specific tolerance to starvation in *Acartia tonsa*. Ajiboye *et al.* (2011) reviewed the list of more than 60 copepod species which have been laboratory raised and the authors stated that this has not encouraged the mass culture of copepods at commercial levels and this could probably be a result of the lack of knowledge about large-scale cultivation. Fahad Alajmi *et al.* (2014) observed the improvement in the reproductive productivity of the tropical calanoid copepod *Parvocalanus crassirostris* through selective breeding. York *et al.* (2014) studied the trophic links in the plankton in the low salinity zone of a large temperate estuary: top-down effects of introduced copepods. Drillet *et al.* (2014a) studied the egg cannibalism in *Acartia tonsa*: effects of stocking density, algal concentration, and egg availability. Drillet *et al.* (2014b) made an attempt on total egg harvest by the calanoid copepod *Acartia tonsa* (Dana) in intensive culture-effects of high stocking densities on daily egg harvest and egg quality. Zhang *et al.* (2014) analysed the possible ways to increase the productivity of the copepod *Acartia tonsa* (Dana) and reported the effects of population density and food concentration. Van der Meeran *et al.* (2014) studied the copepod production in saltwater pond systems.

In India, work on copepod culture is very scanty. Merrylal James and Maartin Thompson (1986) cultured the some species like *Cyclops*, *Oithona*, and *Pseudodiaptomus* and introduced them in mariculture; Santhanam (2002) cultured the cyclopoid copepod *Oithona rigida*; Rajkumar *et al.* (2004) cultured the copepod *Acartia clausi*; Rajkumar

(2006) have successfully cultured the calanoid copepods *Acartia clausi* and *A. erythraea*; Vengadeshperumal *et al.* (2010) have successfully cultured the calanoid copepods such as *Acartia southwelli* and *A. centrura* in laboratory condition; Ananth and Santhanam (2011) cultured harpacticoid copepod, *Macrosetella gracilis* and Ananthi *et al.* (2012) cultured different type of copepods such as *M. gracilis*, *Pseudodiaptomus annandalei*, and *Oithona rigida*. Rajthilak *et al.* (2014) studied the laboratory culture and population growth of brackish water harpacticoid copepod, *Nitocra affinis* (Gurney, 1927) under different temperatures, salinities, and diets. Nandakumar (2014) cultured the harpacticoid copepod *Nitocra affinis* under laboratory scale using different vessels. Dinesh Kumar (2016) cultured the copepod *Oithona rigida* using aquaculture wastewater. Jothiraj and Santhanam (2019) have cultured the calanoid copepod *Nannocalanus minor* in a laboratory small scale level. Ananth and Santhanam (2019) cultured the harpacticoid copepod *Tisbe* sp.

Biochemical composition of copepods

Raymont and Krishnaswamy (1960) estimated the carbohydrate content of some marine zooplankton. The different biochemical parameters such as protein, fat, carbohydrate, and vitamin were recorded in zooplankton by Wickstead (1976); Grabner *et al.* (1982) estimated the concentration of protein, nitrogen, lipid, ash, inorganic ions and amino acids in zooplankton. Klein Breteler and Gonzalez (1988) studied the influence of temperature and food on the lipid content of calanoid copepods. Fraser *et al.* (1989) observed the lipid composition of adult and copepodite stage of *Calanus finmarchicus*, *Pseudocalanus* sp. and *Temora longicornis*. The biochemical composition of copepods was observed by some other workers. Ingole and Parulekar (1995); Ward *et al.* (1996) from *Rhincalanus gigas* and *Calanus simillimus* in *Calanus pacificus* and Miller *et al.* (1998) in *Calanus finmarchicus*; Nanton and Castell (1998) investigated the fatty acid content of harpacticoid copepod, *Tisbe* sp, while Neil (1998) recorded the composition of protein,

lipids, and polysaccharide in *Macrosetella gracilis*; Stottrup *et al.* (1999) examined the fatty acid composition of *Acartia tonsa* and Nanton and Castell (1999) studied the fatty acid profile of *Tisbe sp.* and *Amonardia sp.* Hygum *et al.* (2000) determined the lipid content of *Calanus finmarchicus*. Helland *et al.* (2003) determined the free amino acid and protein content in the planktonic copepod *Temora longicornis* and compared with *Artemia franciscana*; McKinnon *et al.* (2003) observed the biochemical composition of the potential tropical *paracalanoid* copepods; Drillet *et al.* (2006) studied the amino acids and fatty acid composition of *Acartia tonsa*. Sorensen *et al.* (2007) examined some biochemical composition of eggs from neritic calanoid copepods *Acartia tonsa*; Van der Meeren *et al.* (2008) found lipid composition, fatty acids, amino acids, pigments, astaxanthin, vitamin, and ascorbic acids in wild copepod; Guangxing and Donghui (2009) studied the fatty acid composition of *Schmackeria poplesia*. The biochemical composition of copepods collected from the wild species was studied by some workers in Indian waters viz: Madhupratap *et al.* (1979) from Cochin backwaters; Rosamma Stephen *et al.* (1979) from the Laccadive Sea; Goswami *et al.* (1981) from Andaman Sea; Krishnakumari and Nair (1988) from the Arabian Sea; Nandakumar *et al.* (1981) from the central Arabian Sea; Krishnakumari and Achuthankutty (1989) from the Arabian Sea; Sreepada *et al.* (1992) from the Bay of Bengal; Bhat and Wagh (1992) from the Arabian sea. Krishnakumari and Goswami (1993) from northwest Bay of Bengal; Ting *et al.* (2000) noticed the protein content in harpacticoid copepod, *Tigriopus japonicus*; Santhanam and Perumal (2001) have reported the amino acid composition of cultured copepod, *Oithona rigida*. Nageshwara Rao and Krupanidhi (2001) investigated the proximate composition of zooplankton from Andaman and Nicobar islands; Santhanam (2002) analysed the biochemical composition of cultured cyclopoid copepod *Oithona rigida*; Srivastava *et al.* (2006) have reported protein content and amino acid composition of *Brachionus plicatilis*; Rajkumar and Vasagam (2006) estimated the

proximate composition of cultured *Acartia clausi*; Perumal *et al.* (2009) have analyzed the biochemical composition of wild copepods *Acartia spinicauda* and *Oithona similis* from Parangipettai coastal waters in relation to environmental parameters; Jagadeesan *et al.* (2010) analyzed the biochemical composition of wild zooplankton, Vengadeshperumal *et al.* (2010) studied the biochemical composition of laboratory cultured copepod, *Acartia southwelli*, *Acartia centrura*. Ananth and Santhanam (2011) studied the biochemical composition of *Macrosetella gracilis*. Wang *et al.* (2014) analysed lipid and fatty acid composition of zooplankton prey of spiny lobster. Koussoroplis *et al.* (2014) observed the effects of diet and temperature on fatty acid dynamics of *Eudiaptomus gracilis*. Ladhar *et al.* (2014) studied the effects of environmental factors on the fatty acid composition of copepods and *Artemia*. The effects of UV radiation on hatching, lipid peroxidation, and fatty acid composition in the copepod was reported by Won *et al.* (2014). Arndt and Sommer (2014) studied the effect of algal species and concentration on the development and fatty acid composition of two harpacticoid copepods, *Tisbe* sp. and *Tachidius discipules*. Piccinetti *et al.* (2014) reported the use of preserved copepods for sea bream culture and its biochemical composition. The pattern result indicates of taxonomic changes in community composition and phylogenetic differences in EFA content rather than a direct influence of temperature on EFAs in zooplankton (Gladyshev *et al.*, 2011; Gladyshev *et al.*, 2014). Isari *et al.* (2015) found direct effects on copepod vital rates, nor indirect effects, via phytoplankton fatty acid composition, in two copepods *Acartia granii* and *Oithona davisae*. Almén *et al.* (2016) described those female monounsaturated fatty acids were reflected in their eggs, whereas saturated fatty acids were not, and none of them had a significant effect on nauplii production.

Microplastics research in copepod

Amandine Collignon *et al.* (2012) have studied the Neustonic microplastic and zooplankton in the North-Western Mediterranean Sea. Kyun-Woo Lee *et al.* (2013) studied the size-dependent effects of micro polystyrene particles in the marine copepod *Tigriopus japonicus*. The impact of Polystyrene microplastics on feeding, function and fecundity in the marine copepod *Calanus helgolandicus* was observed by Matthew Cole *et al.* (2015). He stated that microplastics impede feeding in copepods, which over time could lead to sustained reductions in ingested carbon biomass. Jung-Hoon Kang *et al.* (2015) studied the potential threat of microplastics to zooplanktivores in the surface waters of the Southern Sea of Korea. Impacts of microplastics on plankton was observed by Vivian (2016) and he concluded that the interactions of microplastics with planktonic organisms will clarify the effects of pollutants.

Adverse effects of microplastics and oxidative stress-induced MAPK/Nrf2 pathway-mediated defense mechanisms in the marine copepod *Paracyclopina nana* was examined by Chang-Bum Jeong *et al.* (2016). The study provides the first insight into the mode of action in terms of microplastic-induced oxidative stress and related signaling pathways in *P. nana*. Matthew Cole *et al.* (2016) stated that microplastics can alter the properties and sinking rates of zooplankton. Rosana Di Mauro *et al.* (2017) studied the abundant plankton-sized microplastic particles in shelf waters of the Northern Gulf of Mexico. Heindler *et al.* (2017) reported the toxic effects of polyethylene terephthalate microparticles and Di (2-Ethylhexyl) phthalate on the calanoid copepod, *Parvocalanus crassirostris*.

Xiaoxia Sun *et al.* (2017) examined the ingestion of microplastics in five different groups of zooplankton in the coastal region of Northern South China. As a result, they found polyester a type of microplastic as a main component with an average length of the 125 μm and 167 μm . The effects of nylon MPs on feeding, lipid accumulation, and moulting in a

cold-water copepod was reported by Matthew Cole *et al.* (2019). The results emphasize that the shape and chemical profile of a microplastic can influence its bioavailability and toxicity. Coppock *et al.* (2019) observed that microplastics alter feeding selectivity and fecal density in the copepod, *Calanus helgolandicus*. Lisbet Sørensen *et al.* (2019) studied the bioavailability and toxicity of micro-plastic on marine copepods under co-exposure conditions. The availability of microalgae in relation with micro-plastic ingestion and their impacts on marine copepod *Pseudodiaptomus annandalei* was studied by Yuqi Cheng *et al.* (2020). Their results suggest that availability of microalgae may increase the ingestion and retention of microplastics in marine copepods. The study of assimilation and impact of microplastics on Arctic *Calanus* copepods was carried out by Rocío Rodríguez-Torres *et al.* (2020) in which it was suggested that the exposure of MP can ground stress that induced the rate of spawning in Arctic copepods.

Jin Soo Choi *et al.* (2020) evaluated the toxicity of microplastic in the marine copepod (*Tigriopus japonicus*) with different sizes and exposure time. The study provides a significant insight into the molecular mechanisms of microplastic-induced noxiousness in the oceanic organism. Review on the effects of microplastics on marine copepods was analyzed by Zhuoan Bai *et al.* (2021). In his appraisal he stated that microplastic ingestion is widespread and it may cause physiological stress in copepods which includes failure of immune responses, metabolic disorders, energy depletion, behavioral alterations, growth retardation, and reproduction disturbance.

Microplastic availability to pelagic amphipods in sub-Antarctic and Antarctic surface waters was studied by Kirstie Jones-Williams *et al.* (2020). The study highlights the need to precede the zooplankton abundance and plastic pollution which act as a potential interaction element in the Antarctic Ecosystem. Effects of microplastics to marine

zooplankton was examined by Zara *et al.* (2020) in which they stated that the rate of risk was high during ingesting aged microplastics from the marine environment.

Effect of microplastics on the swimming behavior of the copepod *Temora turbinata* was studied by Caroline *et al.* (2020). The results suggest that the swimming behavior of *Temora turbinata* was affected by microbeads. Jiaji Sun *et al.* 2021 studied the adverse effect of microplastics from discarded surgical masks on the marine copepod *Tigriopus japonicas*. The results clearly suggest the MPs released from improperly discarded SMs could have a long-term domino effect on coastal marine ecosystems. Raju *et al.* (2021) reported the impact of PS-MPs on major marine phytoplankton and zooplankton

3. Scope and Objectives

Plastic litter in the marine environment is a recent concern from a global environmental perspective. Plastics are the main waste which can be found in the marine environment and 70% of marine debris are the same which plastic, accumulates and persists due to its durable nature. The occurrence of small plastics on marine and coastal environment was first noticed in 1970s but the term microplastic was not used yet then. The effects of large plastics items in marine environment can be noticed as the death and accidents are reported from marine animals due to ingestion, and entanglement of plastics. However, the large portion of plastics found in the marine environment is in the microscopic level. These plastics particles are known as microplastics (MPs). Their size ranges from 1 to 5 mm and belong to the microplastics group. Microplastics are synthetic materials with a high polymer content which are insoluble in water and non-degradable in nature. Microplastics have emerged as a recent threat to the global environment. This study focused on the effect of ingestion and biological effects of microplastics debris on copepods.

Based on the above insight, the present study was aimed to made attempt with the following specific objectives:

- ✚ To study the diversity of marine copepods in Nagapattinam coastal waters.
- ✚ To analyse the morphological and molecular identification of marine copepod *Oithona dissimilis*.
- ✚ To optimize the culture conditions for marine copepod *Oithona dissimilis*.
- ✚ To examine the impact of microplastics ingestion on survival, nauplii production, population density and post embryonic development of marine copepod *Oithona dissimilis*.
- ✚ To assess the impact of microplastics ingestion on nutritional profile of marine copepod *Oithona dissimilis*.

4. Description of the Study Area

Description of the study area

The present investigation was carried out in Bay of Bengal and adjacent Kaduvaiyar estuary, Nagapattinam coast of Tamil Nadu, Southeast India. The freshwater influence is high due to fluctuations in tide and incursion of freshwater during monsoon because the Kaduvaiyar estuary is debouching in the Bay of Bengal. Sampling sites are shown in Fig.1. There are three sampling stations viz; ST-1 (Neritic Zone) 10°45'53.98"N; 79°52'20.29"E), ST-2 Sea Mouth (10°45'54.63"N; 79°51'42.32"E) and ST-3 Kaduvior estuary (10°45'52.80"N; 79°51'7.49") were fixed and monthly samplings were carried out from February 2019 to January 2020.

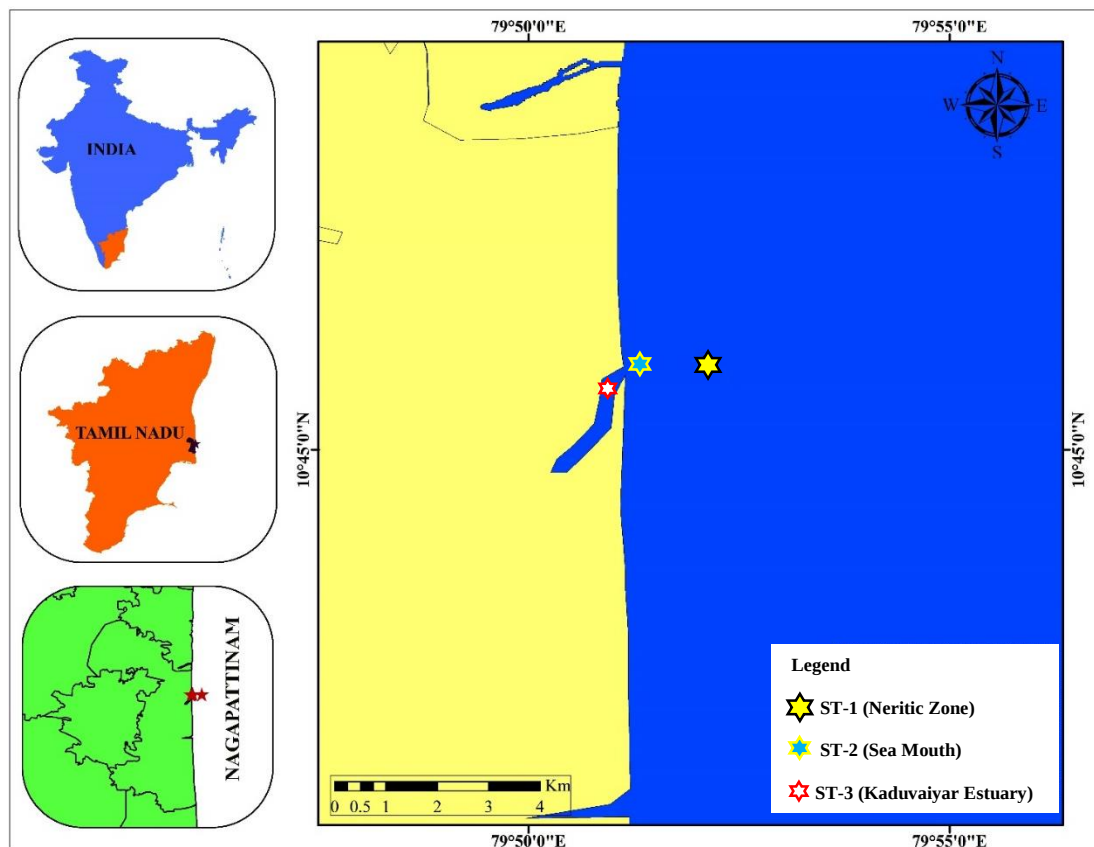


Fig. 1. Map showing the sampling areas in Nagapattinam coastal waters, Southern India

The Nagapattinam coastal waters have been chosen as the sampling area for this study because of the increased anthropogenic activities carried out in the surrounding area in the recent years. The shrimp ponds operated adjacent the estuary adds loads of nutrients into the coastal waters making it an affordable place for a rich diversity of organisms. Moreover, the water quality and biodiversity of the Kaduvaiyar estuary has not been studied in a continuous scale. In particular, the copepod diversity has not been studied in the recent years. Therefore, the present study was made at Nagapattinam coastal waters.

To assess the water quality and copepods distribution, three sampling stations were fixed as follows:

Station-1

Station 1 represent the neritic zone in Bay of Bengal and has a depth of about 15 to 20 m. The bottom is characterized by fine silt (Plate 1).

Station-2

The station 2 is represent the sea mouth and located about 500 m away from station 1. The depth of this station is around 5 to 10 m. The bottom is characterized by sand (Plate 2).

Station-3

The station represent the Kaduvaiyar estuary can be characterized as estuarine zone, as it remains mostly unconnected with other stations. The station received bulk of freshwater during monsoon season. The depth of the station ranged from 1 to 5 m. The bottom of the station is silty clay (Plate 3).



Plate - 1. Station-1 (Neritic Zone)



Plate - 2. Station-2 (Sea Mouth)



Plate - 3. Station-3 (Kaduvaiyar estuary)

Chapter II

5. Ecology of Marine Copepods from Nagapattinam Coastal Waters

Introduction

Copepods population plays a decisive role in the marine food web, by transferring the energy from photosynthetic phytoplankton to higher trophic level organisms. The various physico-chemical and biological processes influence the productivity and community dynamics of copepod within coastal and marine environments. There has been considerable research already focused on the effects of temperature (Austin and Jones, 1974), salinity (Silva *et al.*, 2009; Zervoudaki, 2009), on the spatio-temporal variability of zooplankton (Dragovich and Kelly, 1967; Badylak and Philips, 2008). However, only limited research was carried out on the copepod population dynamics in the Nagapattinam coastal waters (Vengadesh Perumal, 2009). Nagapattinam coast is dominated by fishing activities. This area has been subjected to diverse anthropogenic pressures including land-discharges, degradation of habitats with the subsequent declining of plant and animal populations, and the finally diminishing of fish harvests. Many copepod species have wide distributions but exhibit preferences for specific environmental conditions within their range. There is also a growing body of evidence that production rate and the distribution pattern of copepod may be strongly affected by ozone depletion and the consequent increased UV radiation (Hader *et al.*, 1993, Malloy *et al.*, 1997, Kouwenberg, 1999; Newman *et al.*, 1999, Hwang *et al.*, 2014). The combination of environmental sensitivity, short life histories, and the inability to escape from their surroundings, make the copepods as an excellent indicator of environmental change. The copepod is also an important intermediate component in

estuarine and marine food web, by acting as a tropical link between small organic particles (e.g., detritus and microalgae) and the planktivorous fishes (McLusky and Elliott, 2004; Varadharajan and Soundarapandian, 2013). Knowledge on the spatio-temporal variability of estuarine and marine copepods composition and abundance is a prerequisite to understand the ecosystem dynamics. In marine and estuaries, the temporal variation of environmental conditions largely influence the distribution of copepod species (Dauvin *et al.*, 1998). Eventhough Nagapattinam coastal environment is ecologically and socially important place, only limited information is available about its hydrobiological characteristics. The present investigation pertains to the influence of physico-chemical features on the distribution and abundance of the zooplanktonic-copepods at Nagapattinam coastal waters.

Materials and methods

Sampling and analysis

Monthly samplings of sea water and copepods were made, (Plate 4). The meteorological factors viz; rainfall, atmospheric and surface-water temperature were analysed and chemical features viz; dissolved oxygen, pH, and inorganic nutrients (of water) were measured and recorded. Parameters like atmospheric and surface water temperatures, were measured by using standard mercury filled centigrade thermometer. Salinity was estimated with the help of a Hand Refractometer (ERMA, Japan) and pH was measured using pH meter (Elico Grip). Dissolved oxygen was estimated by adopting the modified Winkler's method (Strickland and Parsons, 1972). For the nutrients analysis, surface water samples were collected in a clean polyethylene bottle and immediately kept in an ice box and transported to the laboratory. At laboratory, the water samples were filtered using a Millipore filtering system (MFS) and analyzed for dissolved inorganic phosphate,

nitrate, nitrite, reactive silicate and ammonia by adopting the standard methods described by Strickland and Parsons (1972), and Jenkins and Medsken (1964).

Copepod (zooplankton) samples were collected from surface water by using the Indian Ocean Standard Plankton net with a mouth diameter of 0.35 m and mesh size of 158 μm . Regular monthly samplings were made by horizontal towing of plankton net for 30 minutes and the collected samples were preserved by using 5% neutralized formalin for identification. Copepods were identified by referring the standard keys of Kasturirangan (1963) and Perumal *et al.* (1998). Quantitative estimate of copepods was carried out by filtering 500 l of water through a bag net of same mesh size and the numerical analysis was carried out by using an inverted microscope (Micros, Austria). Biodiversity indices were calculated by following the standard formulae: Species diversity: $H_1 = -\sum P_i \log P_i$; $I = 1$; (Shannon and Weaner, 1949); richness: $D = 1/C$; $C = \sum P_i^2$; $P_i = n_i/N$ (Gleason, 1922) and evenness: $J' = H'/\log_2 S$ (Pielou, 1966). The methods and unit of expression of physico-chemical and biological parameters used is given in Table 1.

Statistical analyses

Correlation coefficient analysis was carried out to interpret the relationship between copepod population diversity and physico-chemical factors.

Parameters	Abbreviations	Techniques/Instruments used	Unit
Rainfall	RF	Meteorological Department, Nagapattinam	mm
Atmospheric Temperature	AT	Standard Centigrade Thermometer	°C
Surface water Temperature	ST	Standard Centigrade Thermometer	°C
pH	pH	pH meter (Elico Grip)	-
Salinity	SL	Hand Refractometer (ERMA, Japan)	PSU
Dissolved Oxygen	DO	Modified Winkler's titration method (Strickland and Parsons, 1972)	mg/l
Phosphate	PO	Spectrophotometry (Strickland and Parsons, 1972)	µmol L ⁻¹
Nitrate	NA	Spectrophotometry (Jenkins and Medsken, 1964)	µmol L ⁻¹
Nitrite	NI	Spectrophotometry (Strickland and Parsons, 1972)	µmol L ⁻¹
Ammonia	AM	Spectrophotometry (Strickland and Parsons, 1972)	µmol L ⁻¹
Silicate	SI	Spectrophotometry (Strickland and Parsons, 1972)	µmol L ⁻¹
Chlorophyll 'a'	CL	Spectrophotometry (Mantoura and Llewellyn, 1983)	mg/m ³
Copepods collection	-	Horizontal Towing by using IOES plankton net with size of 0.35m mouth diameter, No. 10, 158 µm of bolting silk mesh	-
Copepods identification	-	Davis (1955), Kasturirangan (1963), Smith (1977), Perumal <i>et al.</i> (1998) Santhanam and Perumal (2008)	-
Population Density	PD	Sedgewick counting chamber	ind./l
Species Diversity	SV	Shannon &Weaner (1949)	-
Species Richness	SR	Gleason (1922)	-
Species Evenness	SE	Pielou (1966)	-

Table 1. Techniques used for the analysis of physico-chemical and biological characteristics of Nagappattinam coastal waters sample



Plate 4. Collection of water and copepod samples from Nagapattinam coastal waters

Results

Analysis of physico-chemical characters of Nagapattinam coastal waters

In the present study, the rainfall was recorded in the ranged between 2.7 and 365.4 mm, with the lowest value of 2.7 mm in April 2019 and the highest value of 365.4 mm in November 2019 (Fig. 2). In case of atmospheric temperature, the maximum of 33 °C was recorded in March and June 2019 (at stations 1 & 2) and the minimum of 25°C in December 2019 and January 2020 at station 3 (Fig. 3). The maximum surface water temperature of 29°C was recorded in June and July 2019 at station 1 and the minimum of 24°C was recorded in December 2019 and January 2020 at stations 2 and 1 respectively (Fig. 4).

In the present investigation, a wide range of salinity was recorded among the three stations. In station 1, the maximum of 34 PSU salinity was noticed during May 2019. In station 3, the minimum of 18 PSU was noticed during October 2019 (Fig. 4). At all the three stations, the hydrogen ion concentration (pH) remained alkaline throughout the year. The highest pH value was (8.24) noticed in station 1 during February 2019 and the lowest of 7.9 was recorded in September 2019 at station 3 (Fig. 5). The dissolved oxygen concentration was recorded maximum (5.48mgL⁻¹) during May 2019 at station 1 and minimum of 3.71mgL⁻¹ was recorded during October 2019 at station 2. (Fig. 6)

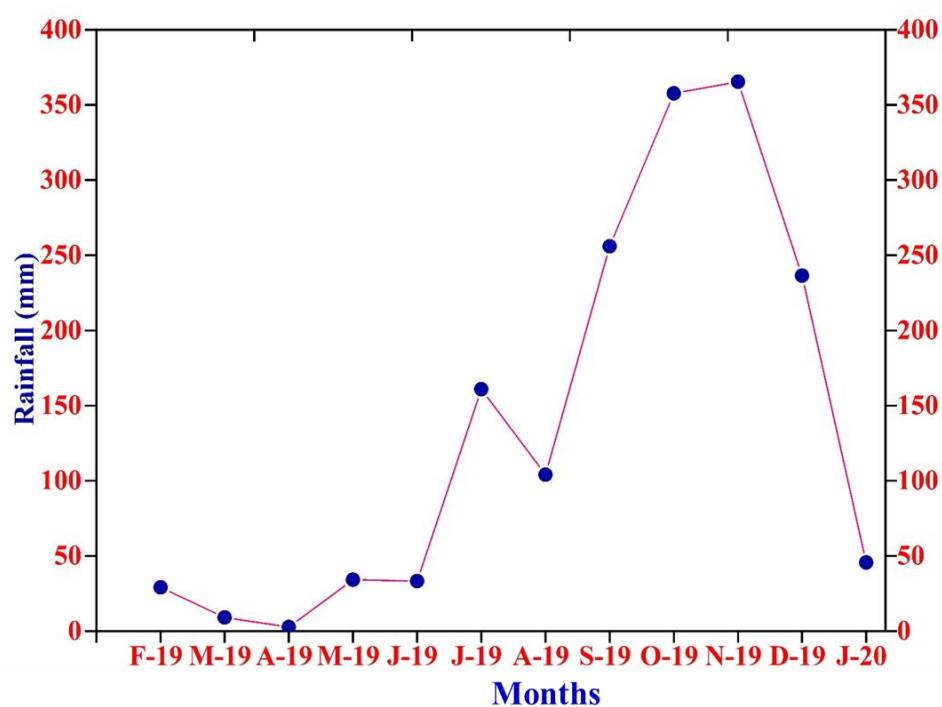


Fig. 2. Seasonal variation of rainfall in Nagapattinam coastal waters

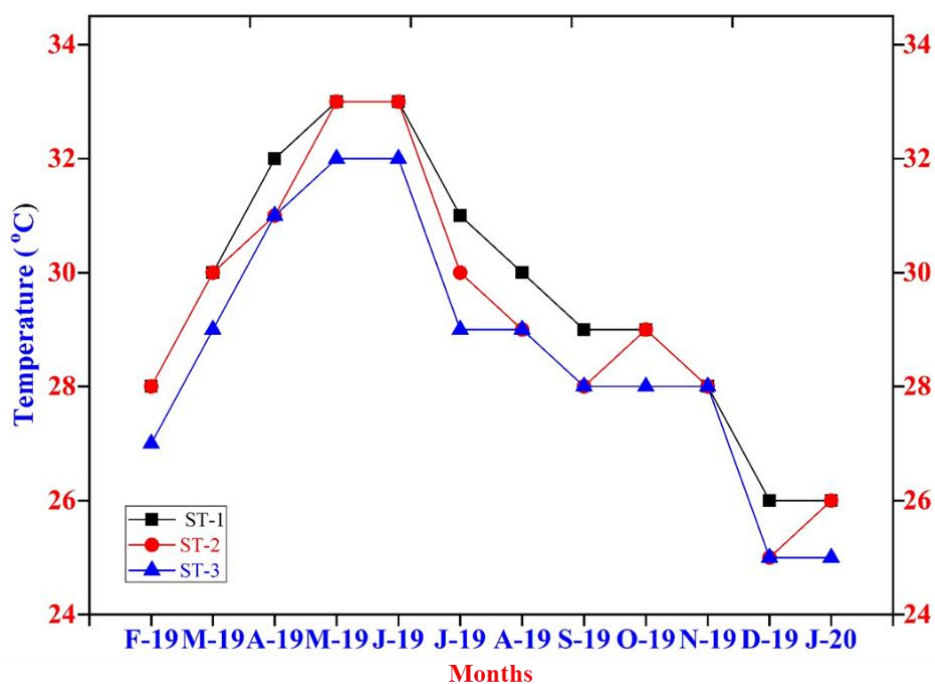


Fig. 3. Seasonal variation of atmospheric temperature in Nagapattinam coastal waters

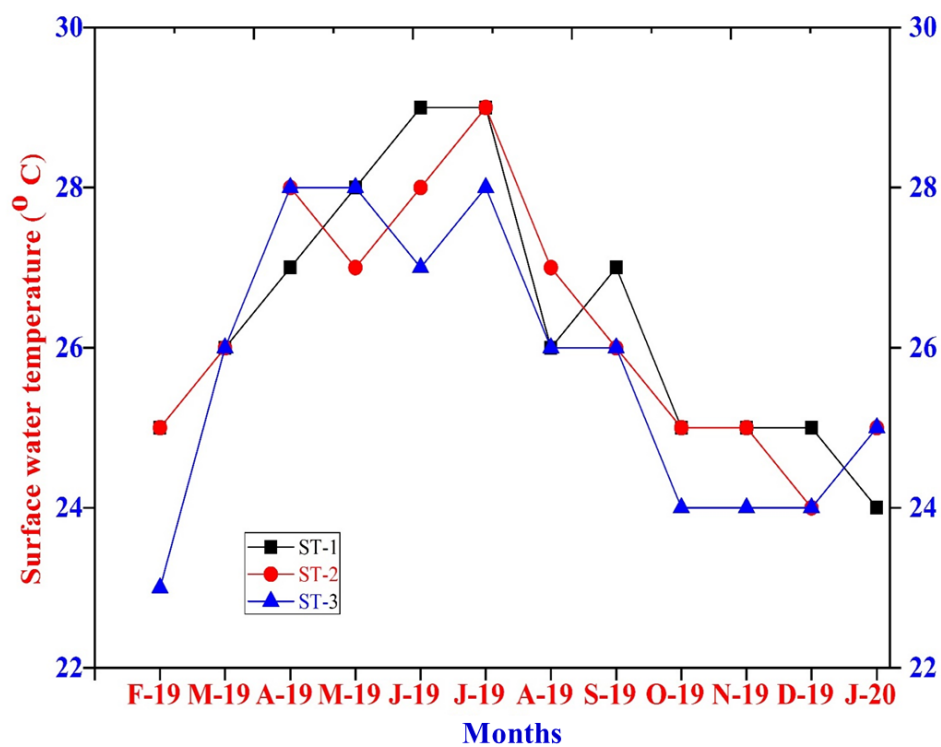


Fig. 4. Seasonal variation of surfacewater temperature in Nagapattinam coastal waters

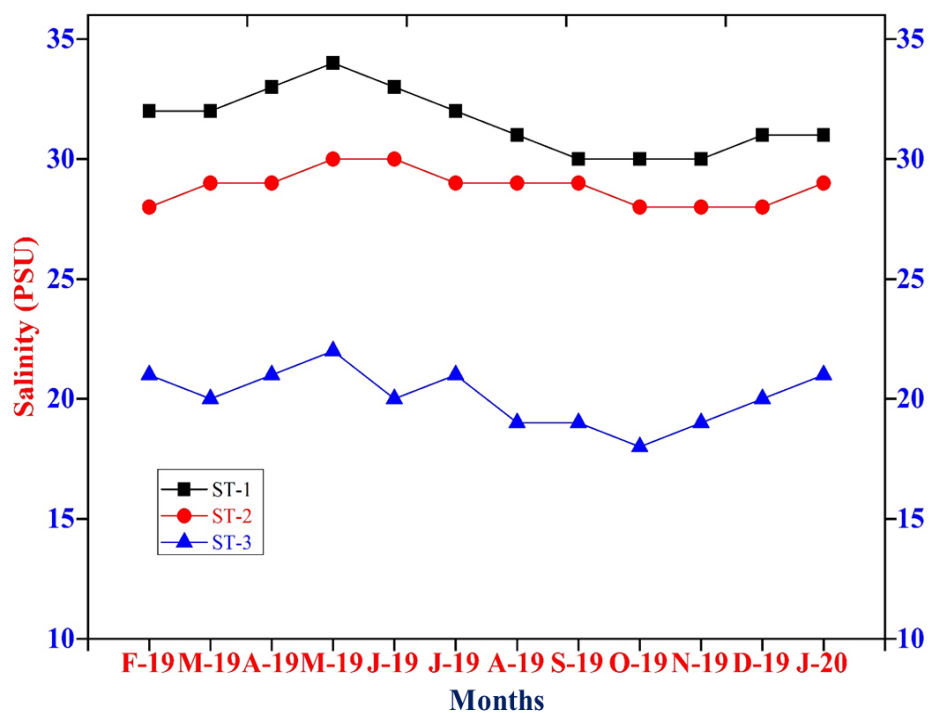


Fig. 5. Seasonal variation of salinity in Nagapattinam coastal waters

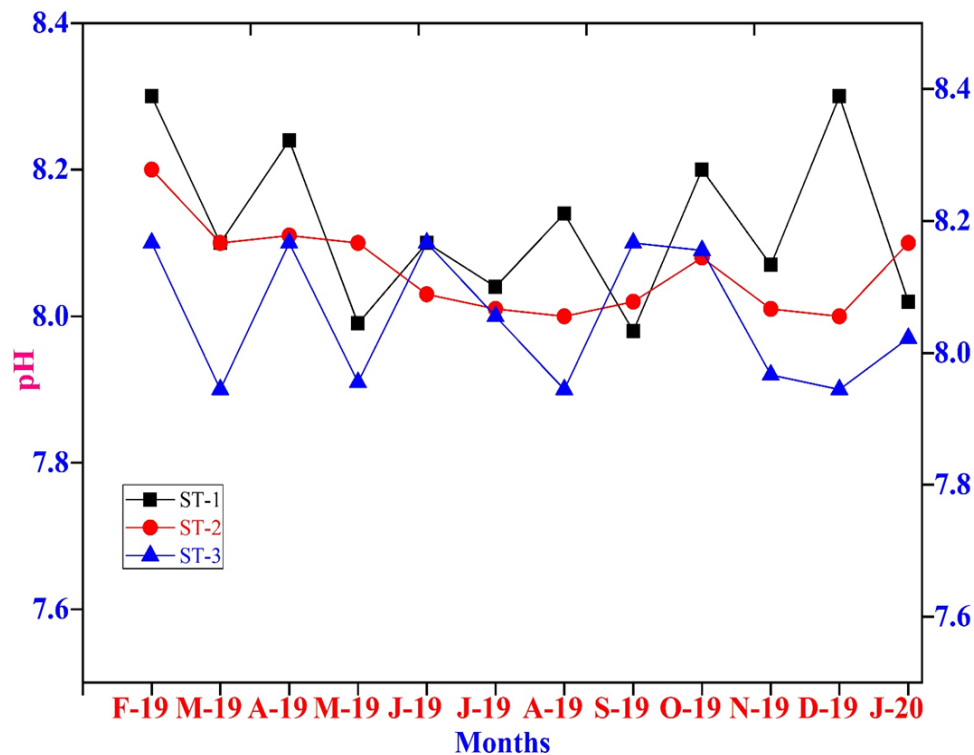


Fig. 6. Seasonal variation of pH in Nagapattinam coastal waters

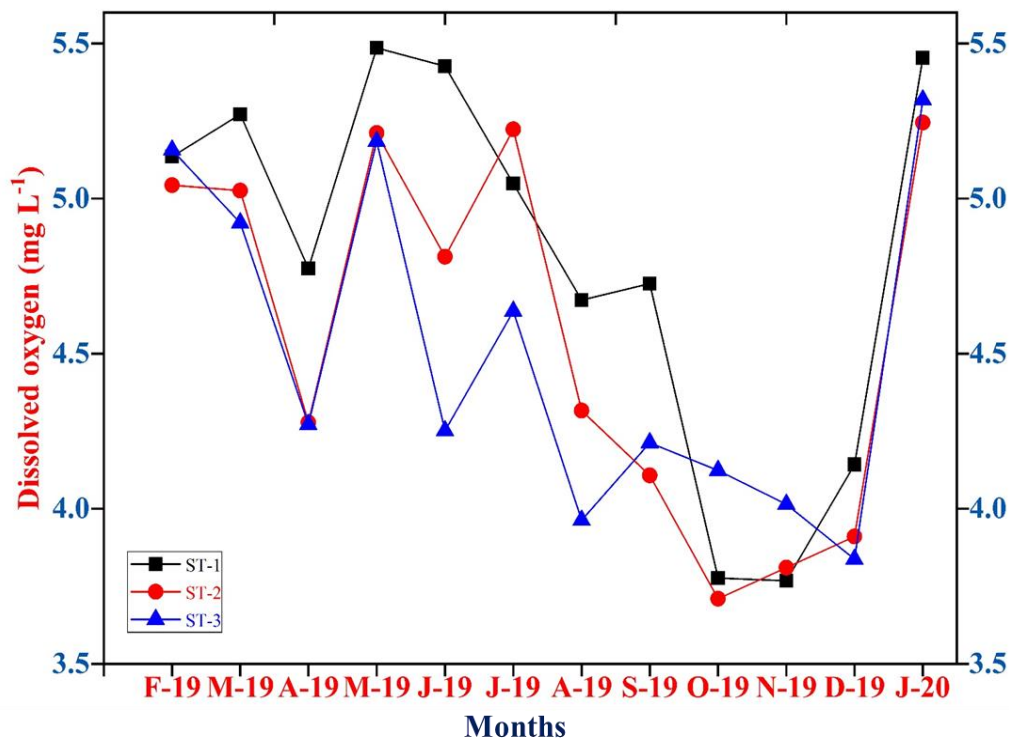


Fig. 7. Seasonal variation of dissolved oxygen in Nagapattinam coastal waters

Analysis of inorganic nutrients

Presently, the inorganic nitrate concentration was found to be vary from 11.11 to 21.82 μmolL^{-1} at station 1. The maximum nitrate (21.82) was recorded in November 2019, and the minimum (11.11) in February 2019, at station 1 (Fig. 7). The nitrite content was ranged between 0.54 and 2.61 μmolL^{-1} . The maximum nitrite value of 2.61 μmolL^{-1} was recorded in May, 2019 and the minimum value (0.54 μmolL^{-1}) in November 2019 at station 1 (Fig. 8). The phosphate content was noticed between 5.65 and 13.14 μmolL^{-1} . The maximum phosphate concentration (13.14 μmolL^{-1}) was noticed during September 2019 at station 1 and the minimum phosphate value (5.65 μmolL^{-1}) in recorded April 2019 (Fig. 9). The silicate concentration was varied from 29.49 to 35.10 μmolL^{-1} . The maximum (35.10 μmolL^{-1}) silicate content was found in June 2019 at station 2 and the minimum of 24.94 μmolL^{-1} was noticed in January 2020 at station 3. (Fig. 10). The ammonia values varied from 0.60 to 1.53 μmolL^{-1} with the maximum (1.53 μmolL^{-1}) in August 2019 at station 1 and the lowest value of ammonia (0.60 μmolL^{-1}) was noticed in June 2019 at station 3 (Fig. 11).

Biological parameters

The chlorophyll 'a' values were found to be range between 0.18 and 0.59 mg L^{-1} (Fig. 12). The maximum value (0.59 mg L^{-1}) was recorded in May 2019 and the minimum value (0.18 mg L^{-1}) was observed in October 2019 at station 3. The copepod population density was ranged between 1492 and 14763 ind. L^{-1} . The maximum population density (14763 ind. L^{-1}) was noticed in June 2019 at station 3 and the lowest population density (1492 ind. L^{-1}) was recorded in February 2019 at station 1. (Fig. 13). The species diversity was varied from 1.46 to 2.99. The maximum diversity (2.99) was observed in May 2019 at station 1 and the minimum (1.46) in January 2020 at station 3. (Fig. 14). The copepod species richness was varied from 0.51 to 0.98. The maximum richness of 0.98 was observed

in the month of May 2019 at station 3. Whereas the lowest species richness (0.51) was observed during January 2020 at station 1. (Fig. 15). The species evenness was varied from 0.82 to 0.98. The maximum evenness was observed during the months of May, June, August and September 2019 at station 3 whereas the minimum evenness (0.82) was noticed during November 2019 at station 1. (Fig. 16). During the one-year study period, totally 38 species of copepods were recorded from Nagapattinam coastal waters; in which the calanoids being the most dominant order (in all the stations), followed by cyclopoida and harpacticoida forms. The identified dominant copepods species are include; *Pontella* sp., *Labidocera pavo*, *Centropages furcatus*, *Nannocalanus minor*, *Pseudodiaptomus aurivilli*, *Rhincalanus* sp., *Lucicutia flavicornis*, *Calanopia* sp., *Nitocra affinis*, *Thermocyclops inversus* and *Dioithona rigida*.

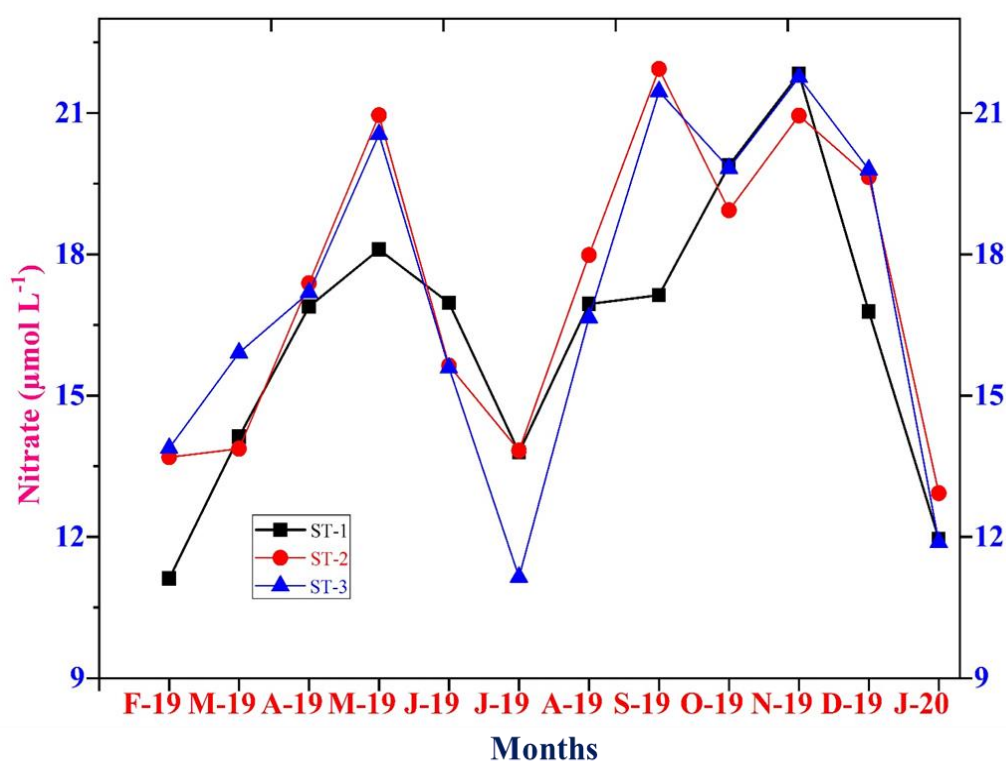


Fig. 8. Seasonal variation of Nitrate in Nagapattinam coastal waters

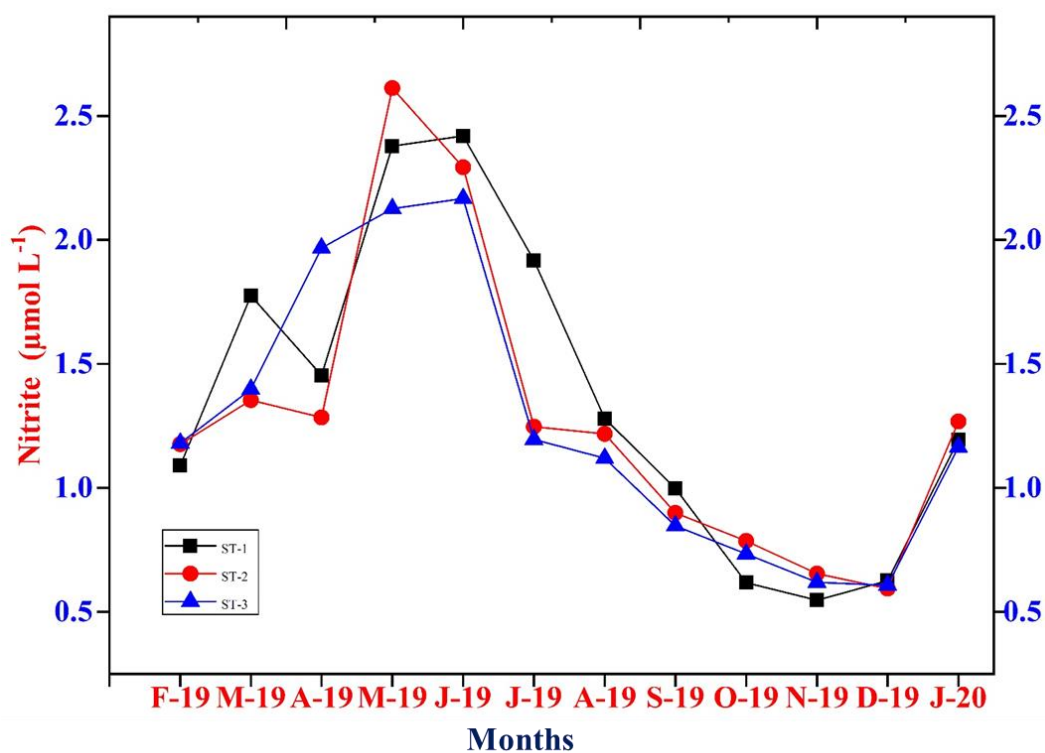


Fig. 9. Seasonal variation of Nitrite in Nagapattinam coastal waters

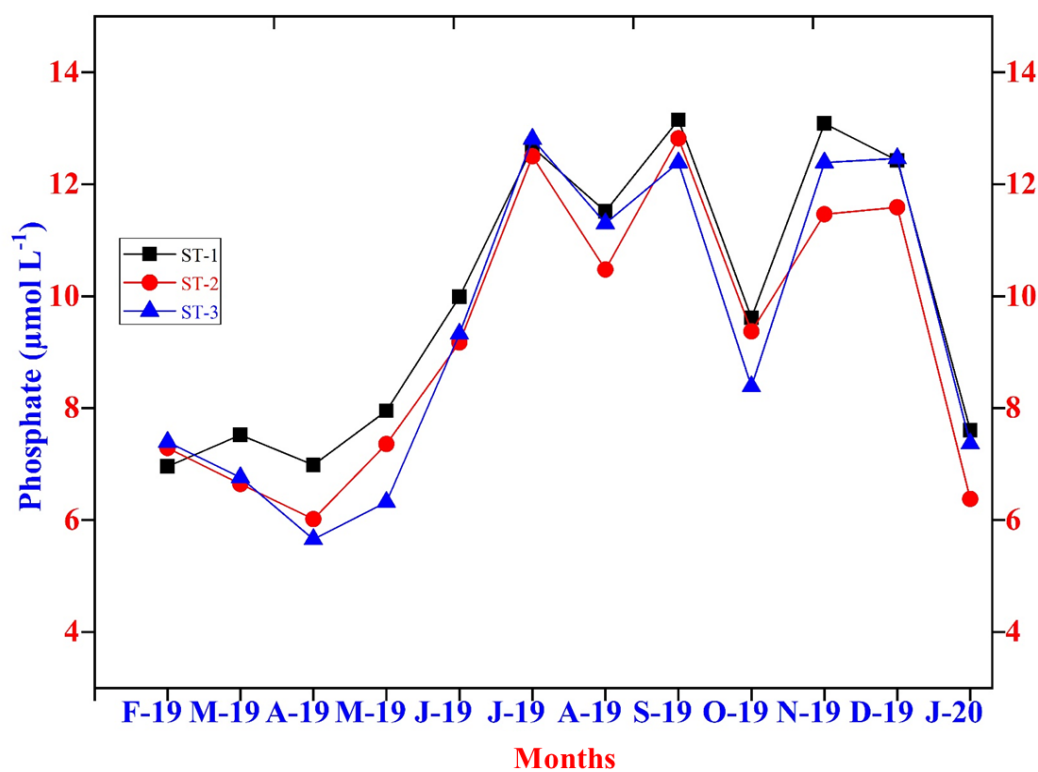


Fig. 10. Seasonal variation of Phosphate in Nagapattinam coastal waters

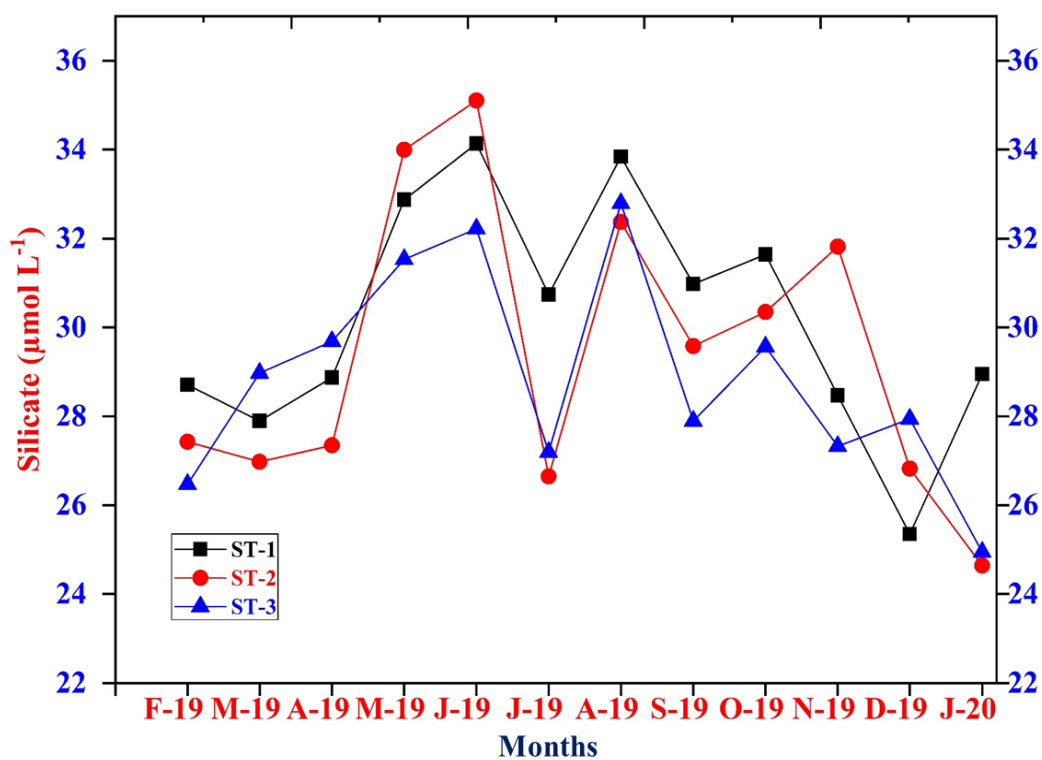


Fig. 11. Seasonal variation of Silicate in Nagapattinam coastal waters

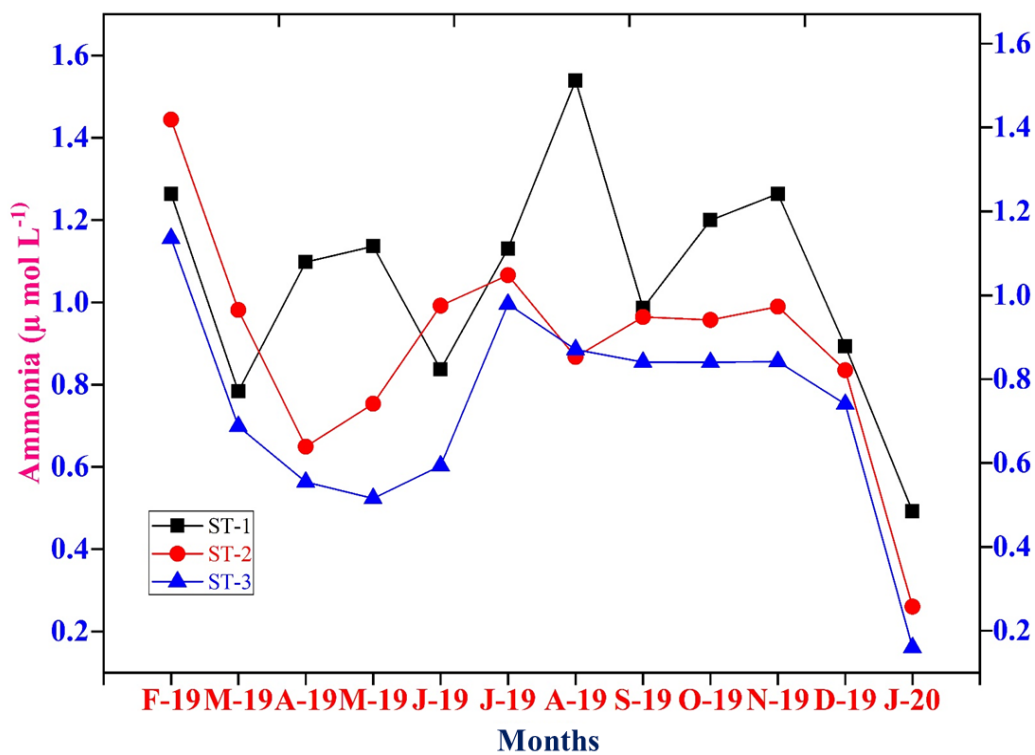
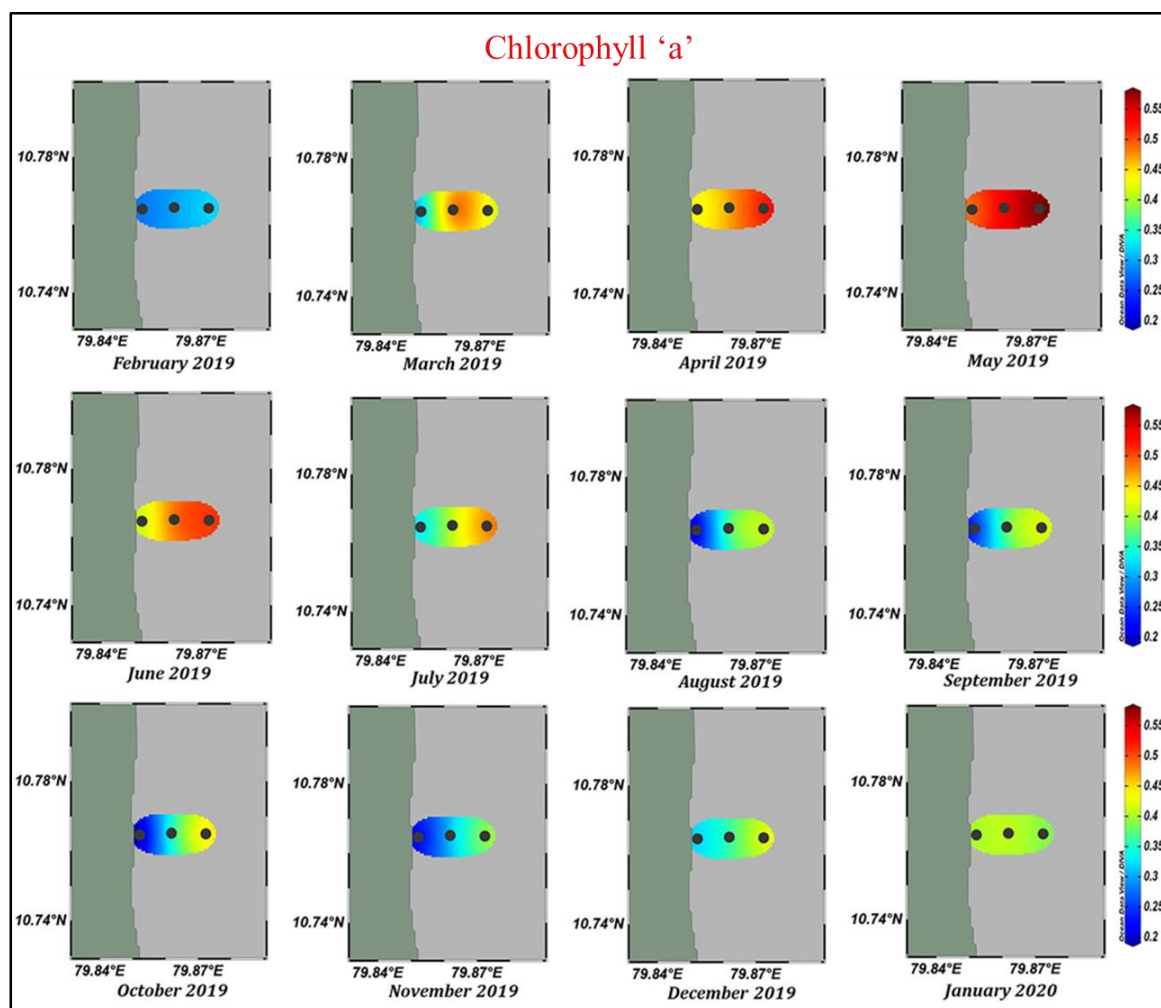
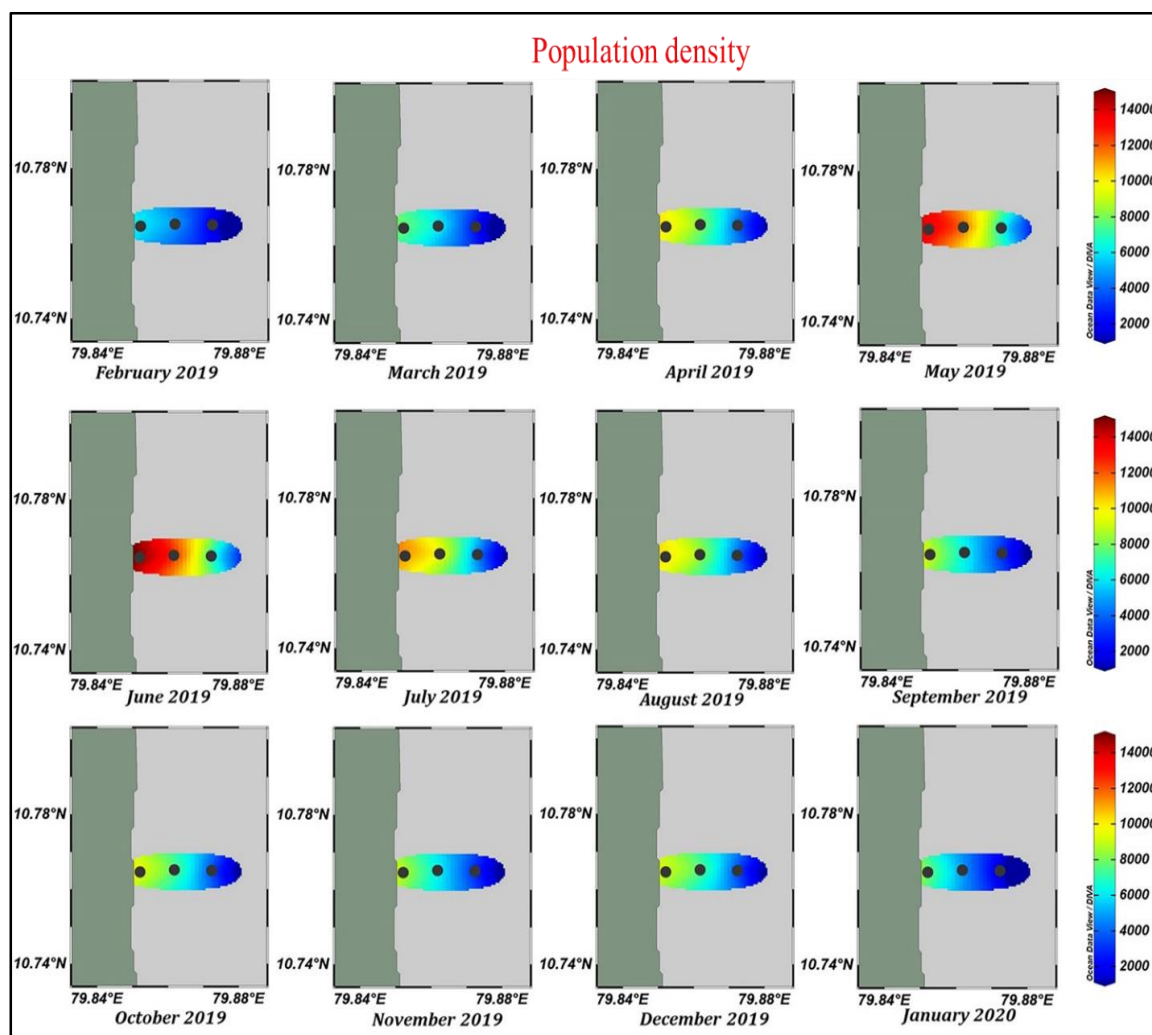


Fig. 12. Seasonal variation of Ammonia in Nagapattinam coastal waters



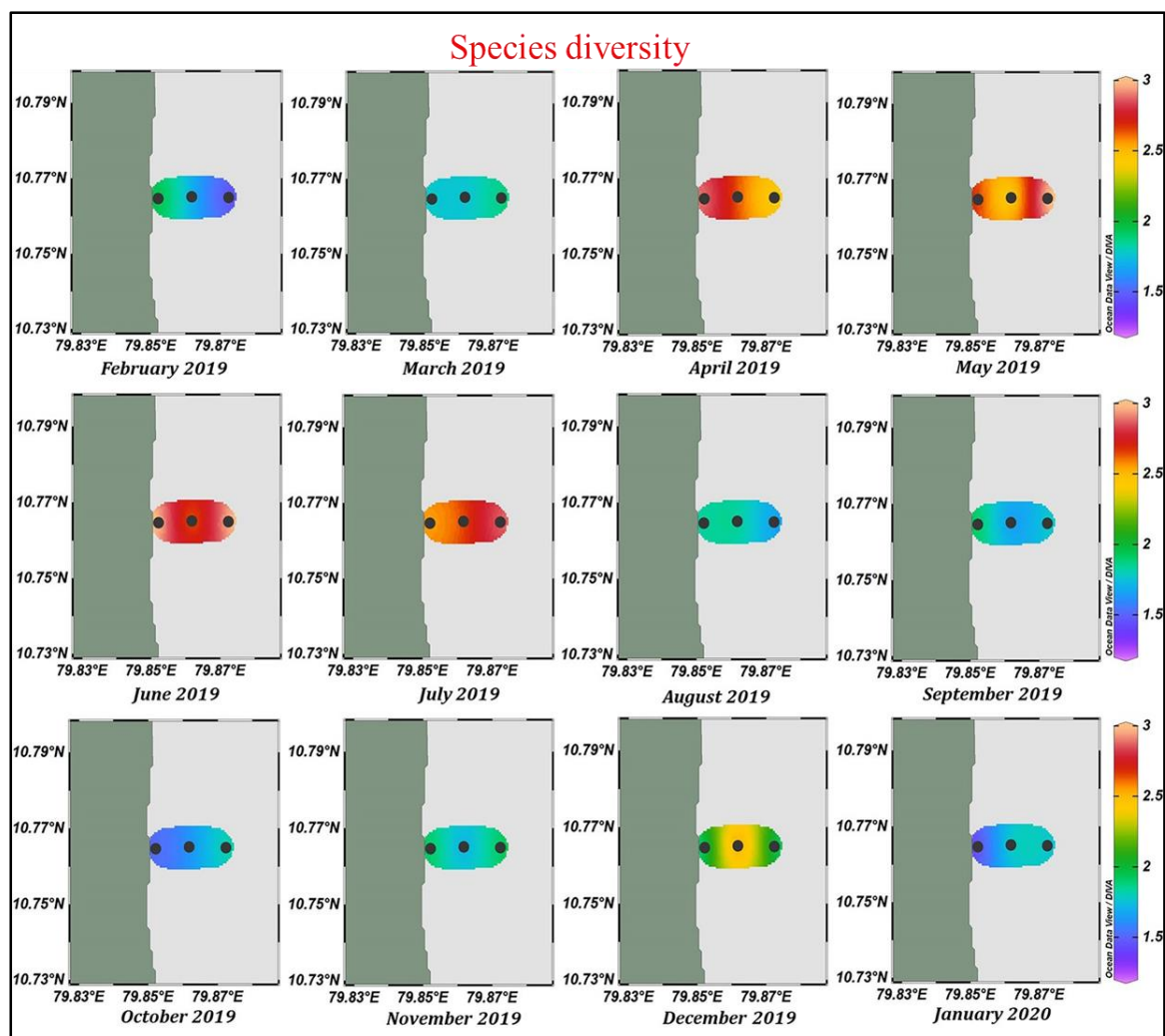
Note : Station 1 ●, Station 2 ●, Station 3 ●

Fig. 13. Seasonal variations of chlorophyll 'a' (mgL^{-1}) in Nagapattinam coastal waters during February 2019 to January 2020



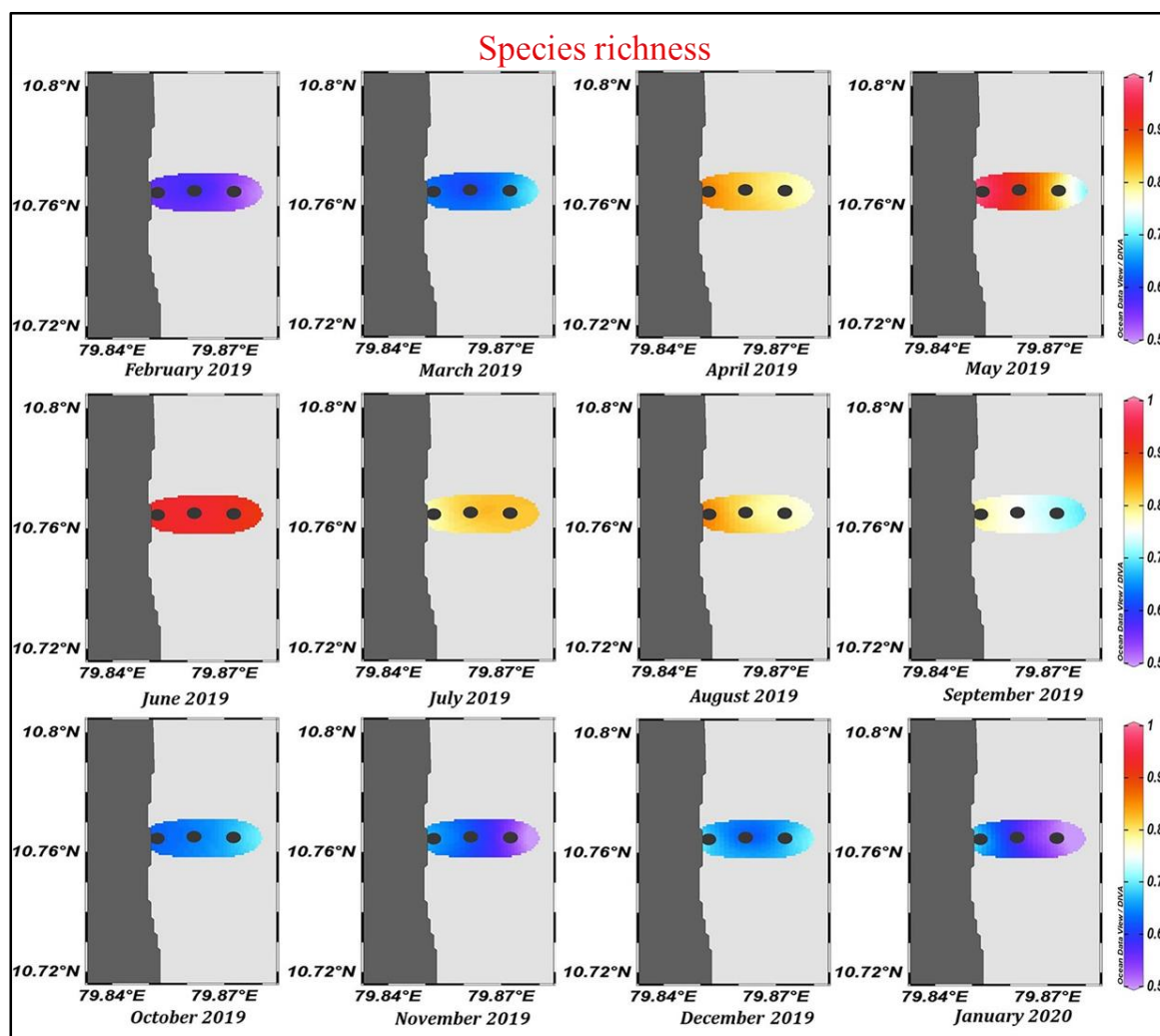
Note : Station 1 ●, Station 2 ●, Station 3 ●

Fig. 14. Seasonal variations of copepods population density (ind. L⁻¹) in Nagapattinam coastal waters during February 2019 to January 2020



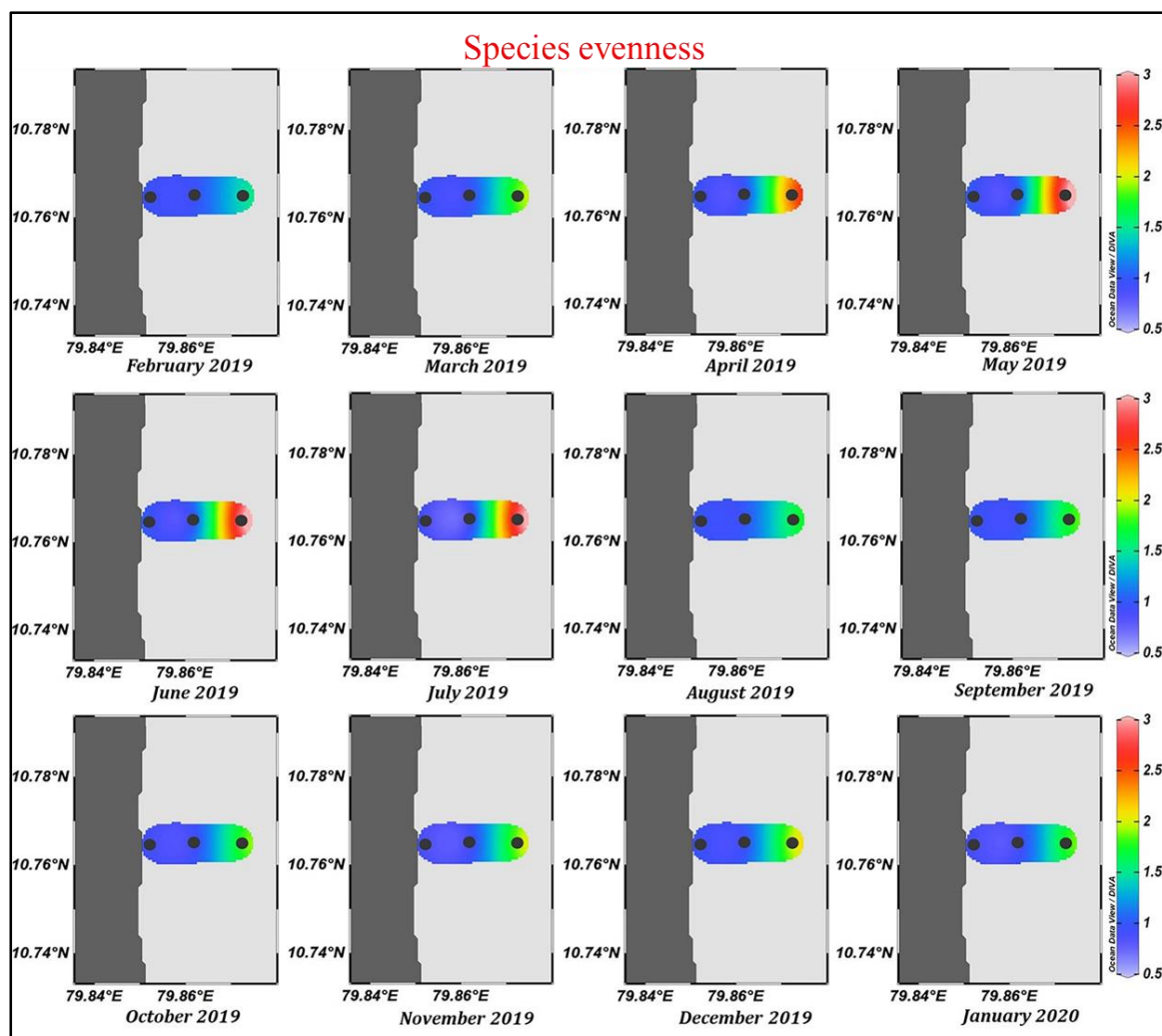
Note : Station 1 ●, Station 2 ●, Station 3 ●

Fig. 15. Seasonal variations of species diversity in Nagapattinam coastal waters during February 2019 to January 2020



Note : Station 1 ●, Station 2 ●, Station 3 ●

Fig. 16. Seasonal variations of species richness in Nagapattinam coastal waters during February 2019 to January 2020



Note : Station 1 ●, Station 2 ●, Station 3 ●

Fig. 17. Seasonal variations of species evenness of copepods in Nagapattinam coastal waters during February 2019 to January 2020

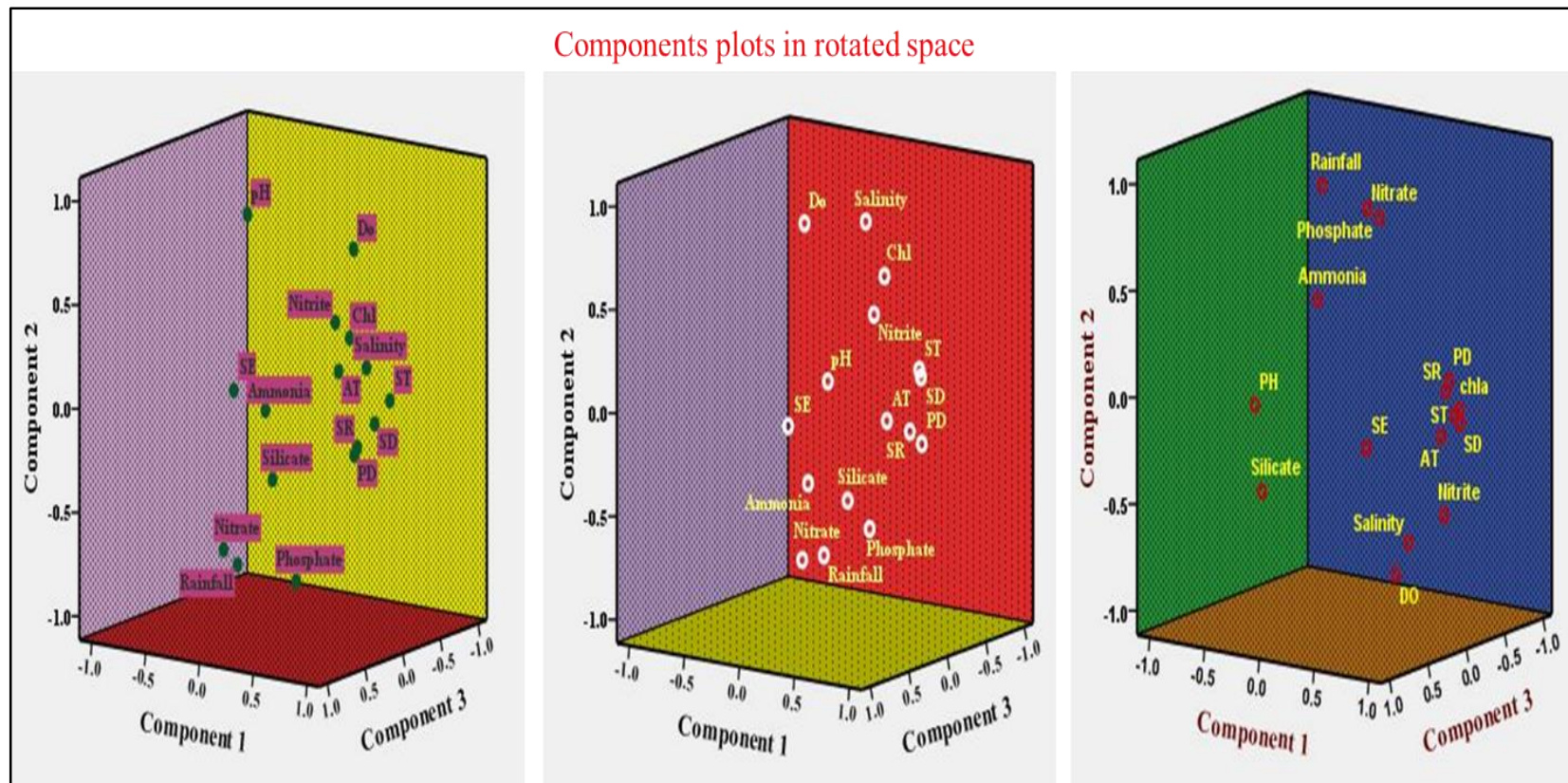


Fig.18. Seasonal variations of components plot in rotated space in Nagapattinam coastal waters during February 2019 to January 2020

	Rain fall	pH	Salinity	AT	ST	DO	PO ₄ ³	NO ₃	NO ₂	NH ₃	SiO ₄	Chl “a”	PD	SD	SR	SE
Rain fall	1.00															
pH	-0.02	1.00														
Salinity	-0.79***	-0.01	1.00													
AT	-0.41*	-0.26	0.72***	1.00												
ST	-0.28	-0.37	0.60***	0.85***	1.00											
DO	-0.87***	-0.36	0.71***	0.40*	0.43*	1.00										
PO₄³	0.72***	-0.23	-0.56**	-0.19	0.14	-0.53**	1.00									
NO₃	0.65***	-0.11	-0.26	0.20	0.06	-0.68***	0.46*	1.00								
NO₂	-0.70***	-0.45*	0.85***	0.81***	0.79***	0.81***	-0.33	-0.24	1.00							
NH₃	0.27	0.24	-0.09	0.23	0.08	-0.42*	0.26	0.39	-0.17	1.00						
SiO₄	-0.12	-0.43*	0.24	0.66***	0.56**	0.27	0.04	0.22	0.53**	0.34	1.00					
Chl- “a”	-0.26	-0.34	0.67***	0.79***	0.73***	0.29	-0.10	0.33	0.69***	-0.05	0.43*	1.00				
PD	-0.14	-0.22	0.59**	0.80***	0.79***	0.21	0.11	0.41*	0.67***	0.16	0.65***	0.82***	1.00			
SD	-0.27	-0.33	0.71***	0.76***	0.84***	0.38	0.00	0.16	0.77	-0.10	0.39	0.88***	0.85***	1.00		
SR	-0.24	-0.14	0.52***	0.81***	0.87***	0.26	0.14	0.20	0.67***	0.14	0.61***	0.76***	0.84***	0.74***	1.00	
SE	-0.46*	0.04	0.36	0.41*	0.38	0.41*	-0.08	-0.13	0.40*	0.19	0.50**	0.20	0.40*	0.87***	0.55**	1.00

Note: *P<0.05; **P<0.02; ***P<0.01; ****P<0.001

Table 2. Correlation matrix among the physico-chemical and biological characteristics of Nagapattinam coastal waters during February 2019-January 2020 at station 1

	Rain fall	pH	Salinity	AT	ST	DO	PO ₄ ³	NO ₃	NO ₂	NH ₃	SiO ₄	Chl “a”	PD	SD	SR	SE
Rain fall	1.00															
pH	-0.02	1.00														
Salinity	-0.79***	-0.01	1.00													
AT	-0.41*	-0.26	0.72***	1.00												
ST	-0.28	-0.37	0.60***	0.85***	1.00											
DO	-0.87***	-0.36	0.71***	0.40*	0.43*	1.00										
PO₄³	0.72***	-0.23	-0.56**	-0.19	0.14	-0.53**	1.00									
NO₃	0.65***	-0.11	-0.26	0.20	0.06	-0.68***	0.46*	1.00								
NO₂	-0.70***	-0.45*	0.85***	0.81***	0.79***	0.81***	-0.33	-0.24	1.00							
NH₃	0.27	0.24	-0.09	0.23	0.08	-0.42*	0.26	0.39	-0.17	1.00						
SiO₄	-0.12	-0.43*	0.24	0.66***	0.56**	0.27	0.04	0.22	0.53**	0.34	1.00					
Chl- “a”	-0.26	-0.34	0.67***	0.79***	0.73***	0.29	-0.10	0.33	0.69***	-0.05	0.43*	1.00				
PD	-0.14	-0.22	0.59**	0.80***	0.79***	0.21	0.11	0.41*	0.67***	0.16	0.65***	0.82***	1.00			
SD	-0.27	-0.33	0.71***	0.76***	0.84***	0.38	0.00	0.16	0.77	-0.10	0.39	0.88***	0.85***	1.00		
SR	-0.24	-0.14	0.52***	0.81***	0.87***	0.26	0.14	0.20	0.67***	0.14	0.61***	0.76***	0.84***	0.74***	1.00	
SE	-0.46*	0.04	0.36	0.41*	0.38	0.41*	-0.08	-0.13	0.40*	0.19	0.50**	0.20	0.40*	0.87***	0.55**	1.00

Note: *P<0.05; **P<0.02; ***P<0.01; ****P<0.001

Table 3. Correlation matrix among the physico-chemical and biological characteristics of Nagapattinam coastal waters during February 2019-January 2020 at station 2

	Rain fall	pH	Salinity	AT	ST	DO	PO ₄ ³	NO ₃	NO ₂	NH ₃	SiO ₄	Chl- “a”	PD	SD	SR	SE
Rain fall	1.00															
pH	-0.02	1.00														
Salinity	-0.74***	-0.01	1.00													
AT	-0.36	0.18	0.11	1.00												
ST	-0.46**	0.01	0.39	0.75***	1.00											
DO	-0.61***	-0.02	0.68***	0.00	0.11	1.00										
PO ₄ ³	0.66***	-0.18	-0.42*	-0.31	-0.16	-0.62***	1.00									
NO ₃	0.57***	-0.11	-0.59***	0.11	-0.16	-0.52**	0.19	1.00								
NO ₂	-0.78***	0.18	0.55***	0.80***	0.73***	0.38	-0.62***	-0.23	1.00							
NH ₃	0.36	0.18	-0.30	-0.04	-0.31	-0.31	0.45*	0.09	-0.40	1.00						
SiO ₄	-0.19	-0.06	-0.26	0.75***	0.46**	-0.35	-0.11	0.29	0.52	0.00	1.00					
Chl- “a”	-0.69***	-0.02	0.79***	0.43*	0.63***	0.49**	-0.52**	-0.25	0.81***	-0.65***	0.13	1.00				
PD	-0.15	0.02	0.07	0.75***	0.70***	-0.14	0.02	0.10	0.65***	-0.27	0.68***	0.52**	1.00			
SD	-0.42*	0.26	0.47**	0.79***	0.74***	-0.03	-0.14	-0.06	0.78***	-0.05	0.46*	0.66***	0.73***	1.00		
SR	-0.33	-0.02	0.19	0.75***	0.82***	-0.10	-0.05	0.13	0.70***	-0.33	0.71***	0.58***	0.87***	0.73***	1.00	
SE	-0.48**	0.08	0.03	0.37	0.22	-0.01	-0.25	0.19	0.44*	0.02	0.60***	0.22	0.23	0.27	0.43*	1.00

Note: *P<0.05; **P<0.02; ***P<0.01; ****P<0.001

Table 4. Correlation matrix among the physico-chemical and biological characteristics of Nagapattinam coastal waters during February 2019-January 2020 station-3.

S. No.	Species Name	ST-1	ST-2	ST-3
Calanoida				
1.	<i>Pontella fera</i>	+	+	-
2.	<i>Pontella dane</i>	+	+	-
3.	<i>Pontella securifer</i>	+	+	-
4.	<i>Pontella</i> sp.	+	+	+
5.	<i>Pontella diagonails</i>	+	+	-
6.	<i>Pontella spinipes</i>	+	+	-
7.	<i>Centropages furcatus</i>	+	+	-
8.	<i>Centropages typicus</i>	+	+	-
9.	<i>Candacia braydi</i>	+	+	-
10.	<i>Nannocalanus minor</i>	+	+	-
11.	<i>Acrocalanus gibber</i>	+	+	-
12.	<i>Acartia spinicauda</i>	+	+	-
13.	<i>Acartia tonsa</i>	+	+	-
14.	<i>Acartia clausi</i>			
15.	<i>Acartia</i> sp.			
16.	<i>Temora turbinata</i>	+	+	-
17.	<i>Tortanus gracilis</i>	+	+	-
18.	<i>Tortanus barbatus</i>	+	+	-
19.	<i>Labidocera acuta</i>	+	+	-
20.	<i>Labidocera minuta</i>	+	+	+
21.	<i>Temora longicornis</i>	+	+	-
22.	<i>Pseudodiaptomus aurivilli</i>	+	+	+
23.	<i>Paracalanus parvus</i>	+	+	-
24.	<i>Eucalanus elongatus</i>	+	+	-
25.	<i>Rhincalanus</i> sp.	+	+	+
26.	<i>Lucicutia flavicornis</i>	+	+	+
27.	<i>Calanopia</i> sp.	+	+	+
Harpacticoida				
28.	<i>Euterpina acutifrons</i>	+	+	-
29.	<i>Nitocra affinis</i>	+	+	+
30.	<i>Metis jousseaumei</i>	+	+	-
31.	<i>Microsetella gracilis</i>	+	+	-
Cyclopoida				
32.	<i>Oithona brevicornis</i>	+	+	-
33.	<i>Oithona dissimilis</i>	+	+	-
34.	<i>Oithona spinirostris</i>	+	+	-
35.	<i>Dioithona rigida</i>	+	+	+
36.	<i>Thermocyclops inversus</i>		+	+
37.	<i>Corycaeus danae</i>	+	+	-
38.	<i>Oncaea venusta</i>	+	+	-

Table 5. List of copepod species recorded in Nagapattinam coastal waters during February 2019-Januvary 2020



Calanopia sp.



Acartia spinicauda



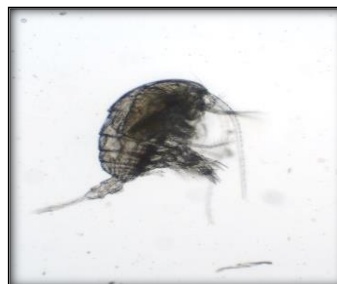
Pontella fera



Pontella acuta



Labidocera euchaeta



Acrocalanus gibber



Pontella sp.



Labidocera minuta



Nannocalanus minor

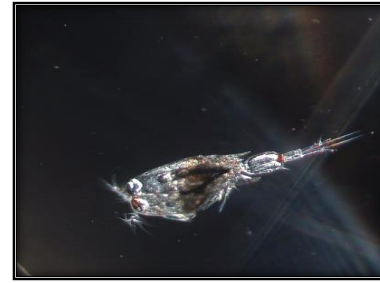
Plate 5. Microscopic images of some calanoid copepods recorded in Nagapattinam coastal waters during Febuary 2019-January 2020



Dioithona rigida



Oithona dissimilis



Corycaeus Sp.



Tigriopus brevicornis



Microsetella gracilis



Nitocra affinis



Euterpina acutifrons

Plate 6. Microscopic images of some cyclopoid and harpacticoid copepods recorded in Nagapattinam coastal waters during Febuary 2019-January 2020

Variables	Station-1				Station-2				Station-3			
	Factor 1	Factor 2	Factor 3	Factor 4	Factor 1	Factor 2	Factor 3	Factor 4	Factor 1	Factor 2	Factor 3	Factor 4
Rainfall		.904			-.409	-.839				-.819		
pH			.850			.869						.777
Salinity	.725	-.628			.800					.877		
AT	.914				.785				.827			
ST	.842				.905				.862			
DO		-.889			.426	.717				.861		
PO ₄ ³		.774				-.889				-.688	-.558	
NO ₃		.835				-.645	.647			-.707		
NO ₂	.755	-.556			.731	.492			.718	.558		
NH ₃		.503	.544	.417				.965		-.417		.709
SiO ₄		-.424	.653		.435		.761		.636		.615	
Chl “a”	.936				.725			-.422	.538	.683		
PD	.929				.882				.924			
SD	.913				.764				.892			
SR	.854				.915				.918			
SE				.789			.798				.816	
Eigen value	6.462	4.152	1.927	1.488	6.772	3.696	1.774	1.287	6.858	3.578	1.432	1.344
% Variance	40.390	25.952	12.044	9.297	42.326	23.0967	11.088	8.041	42.863	22.361	8.951	8.401
Cumulative	40.390	66.342	78.386	87.683	42.326	65.424	76.511	84.552	42.863	65.224	74.175	82.576

Table-6. R mode Varimax sorted factor analysis of physico-chemical and biological parameters of Nagapattinam coastal waters.

Statistical analyses

The obtained data were subjected to statistical analysis to understand the influence of various parameters on the productivity of copepods (zooplankton). The statistical approaches such as Principal Component Analysis (PCA), have been used by several investigators for deriving the significance of specific parameters among the data generated (Helena *et al.*, 2000; Singh *et al.*, 2004 and Shirodkar *et al.*, 2009). Rotation mode (sorted) factor analysis resulted in Eigen values, percentage of variance and cumulative percentage for total stations, allowing inter parameter relation and variation at one station to another stations. Factor analyses of the present data set of the Nagapattinam coastal waters were further sorted by contribution of less significant variables (<0.4 factor score). A varimax rotation of different factors with factor loading was calculated using Eigen value greater than 1 and sorted by the results having for copepod (zooplankton) density and physico-chemical parameters were made; for hydrological values greater than 0.4, based on significant influence (Sahu *et al.*, 1998). The factor was analyzed as the per Liu *et al.* (2003), who categorized the factor loadings as “strong”, “moderate” and “weak” consistent to absolute loading values of >0.75 as “strong”, of $0.75-0.5$ as “moderate” and of $0.50-0.40$ as “weak”. The R-mode factor analyses were implemented on the assigned data set. For the data interpolation, the Eigen values which contains greater than 1 is considered. Different physicochemical (Rainfall, atmospheric temperature, surface water temperature, pH, dissolved oxygen NO_3 , NO_2 , PO_4 , SiO_4 and NH_3).

Biological parameters (chlorella ‘a’, population density, diversity, evenness and richness) was R-mode factor rotation. The data set of the R-mode factor analyses have yielded 1, 2, 3 and 4 factors at station 1, 2 and 3. The highest (87%) cumulative values was recorded at station 1 and lowest (42.86%) at station 3. 87% of cumulative values were

recorded in station 1. The ordination of the variables against the components is shown in Fig. 17 and the factor loadings for the variables are presented in Table 6.

The correlation was subjected to physico-chemical and biological variables were relation to Station and months. The positive values (+) greater than 0.5 termed as strong positive and negative (-) values greater than 0.5 termed as strong negative. The lower values lesser than 0.5 termed as weak. In Station 1, positively correlated with all major nutrients like rainfall, salinity atmospheric temperature, surface temperature, nitrite, nitrate, chlorella 'a' population density, species diversity, species richness, and species evenness; negatively correlated with dissolve dissolved oxygen, phosphate, silicate and ammonia. In station 2, rainfall, phosphate and nitrate negatively correlated; salinity positively correlated with atmospheric temperature, surface water temperature, pH, nitrite, silicate, and chlorella 'a', population density, species diversity, species richness and species evenness. In station 3, positive correlation was observed between atmospheric temperature, surface water temperature pH, nitrite, silicate, chlorella a, population density, species diversity, species richness and evenness. Negative correlation was observed between nutrients, rainfall, phosphate, nitrate, nitrite and ammonia and salinity. The detailed correlation matrix was given in Tables 2, 3 and 4.

Discussion

The Southeast coast of India is being largely influenced by the northeast monsoon-rainfall (Perumal, 1993). Rainfall plays a main role in the hydrographical changes in any estuarine and other coastal environments (Montagna *et al.*, 2018). The presently recorded variations in the water quality parameters including inorganic nutrients are mainly influenced by the seasonal impact of rainfall as discussed earlier by Santhanam and Perumal (2003). The atmospheric temperature seems to be increasing all over the summer and which

gradually decreasing throughout the monsoon period. The maximum atmospheric temperature was recorded during March and June 2019 might be due to clear sky and minimum was recorded in December 2019 and January 2020 might be due to cloudy sky. The surface water temperature was reduced seemingly during rainy season due to the strong land sea breeze and precipitation (Rajkumar *et al.*, 2009) and increased during summer with the influence of increased solar radiation as reported earlier by Santhanam and Perumal (2003) and Ajithkumar *et al.* (2006). During the present study, the maximum surface water temperature was noticed in June and July 2019 and minimum in December 2019 and January 2020.

The salinity is considered to be a major influencing factor in the distribution and spatio-temporal variation of marine organisms due to the dilution and evaporation of waters and such changes inturn largely affect the floral and faunal diversity in the coastal ecosystems (Balasubramanian and Kannan, 2005; Sridhar *et al.*, 2006; Santhanam *et al.*, 2019). The presently recorded maximum summer salinity values could be due to the increased high temperature and evaporation, low rainfall and the interference of neritic water towards the estuary, as stated by earlier workers (Asha and Diwakar 2007; Perumal *et al.*, 2008; Rajkumar *et al.*, 2009; Sridhar *et al.*, 2010). The salinity was found to be low during monsoon season that could have been due to the enormous freshwater influx (Saravanakumar *et al.*, 2007). In the present study, salinity was recorded higher (33 PSU) during May 2019 and minimum (19 PSU) during October 2019.

In the present study, the hydrogen-ion concentration (pH) remained alkaline at all the stations with maximum during summer season might be due to high phytoplankton production and the minimum during monsoon season might be due to low primary production. The dissolved oxygen consumption and production could have been largely

influenced by the algal biomass, light intensity and water temperature. Presently, the maximum dissolved oxygen concentration was recorded in the month of May which might have been due to the influence of heavy freshwater discharges after the monsoonal rainfall (Rajasekar, 2003). Saravanakumar *et al.* (2008) have stated that oxygen solubility varies inversely with salinity and water temperature. The recorded low level of dissolved oxygen concentration during the month of October 2019 could have been due to high rate of organic decomposition and the entry of agricultural runoff, as reported earlier by Sachidanandamurthy and Yajurvedi (2006).

The type and concentration of nutrients availability have a vital impact on the growth, reproduction and prevalence of the marine organisms. Distribution of nutrients is mainly based on the tidal condition and freshwater inflow from terrestrial runoff (Ashok Prabu *et al.*, 2005). Nitrite, which is an intermediate oxidation state between ammonia and nitrate, can emerge as a transient species by the oxidation of ammonia or by the reduction of nitrate (Sathpathy *et al.*, 2010). In general, most of the estuarine waters have relatively high concentration of dissolved inorganic phosphorus and nitrogen. The presently recorded high values of phosphate was due to the settled dead organic matter from top and deposition of sand and the leached of phosphate from sediments, to the overlying water. The increased fertilizers usage, detergents and discharge of domestic sewage might play an important role in contributing to the intense loading of phosphorus in the sediment (Vasanth, 2010). The recorded decreased phosphate value in April 2019 could be attributed to the reduced freshwater flow, high salinity and its utilization by phytoplankton (Rajasegar, 2003). The major contribution of ammonia input into the coastal waters could be from land runoff, zooplankton excretion, or demineralization of organic matter (Ketchum, 1962). The recorded higher concentration during the rainy season could be attributed to the inflow into

the coastal waters, as has been already reported by Burton (1970). The presently found increased level of silicate might be due to the land runoff, carrying the silicate leach out from the rocks and the low values of silicate was recorded during January 2020.

Copepods, being the dominant component of any given zooplankton community, their species diversity is used as an index in all biological monitoring studies to characterize the quality of a water bodies (Gajbhiye *et al.*, 1981). Zooplankton is known to prefer only selected habitats and hence their distribution may vary with species to species and in relation to seasons (Lalli and Parsons, 1997).

In the present investigation, 38 species of copepods was recorded from the Nagapattinam coastal waters. Several investigators including Saravanakumar *et al.* (2007) have recorded 33 species of copepods in the Gulf of Kachchh, Gujarat coast. Vengadesh Perumal *et al.* (2009) reported 43 species of copepods from the Kaduviyar estuary. Santhosh Kumar and Perumal (2011) have recorded 24 species of copepods in the Ayyampattinam coast and Santhi and Ramanibai (2011) recorded 35 species of copepods from Chennai coast. Santhanam *et al.* (2012) recorded 85 species of copepods from Vellar estuary. Madhupradap (1986) has reported that the east coast of India receives huge amount of sewage and industrial wastes and as a result the plankton diversity was decreased in the nearshore coastal waters. Very recently, Santhanam *et al.* (2019) have reported nearly 80 species of copepods from Muthupettai mangrove waters, Southern India. The recorded high population densities might be due to the rationally balanced environmental condition, which occurred during those seasons and great neritic elements presence from the adjacent sea too could have contributed to the maximum density of copepods. Salinity is also a major factor influencing the distribution and abundance of copepods (Padmavathi and Goswami, 1996). The recorded low species diversity in monsoon season could be attributed to the freshwater

influx and decreased salinity. As the estuaries have been called as the "Nurseries of the Sea", the proper monitoring of diversity and ecology of copepod fauna in Nagapattinam coastal water is essential in order to enrich many species of fishes, benthos and birds that depend on this estuary for food and nesting areas.

The recorded lowest copepod density during monsoon season could be ascribed to heavy input of freshwater during which only a few species of copepod have been reported. Similar monsoonal minimum population was reported in other waters (Perumal *et al.* 2008; Santhanam *et al.* 2012). The population density of marine copepod was lowest with salinity changes as evinced by the high copepod density that was during summer season due to increased salinity and phytoplankton population as reported by Madhupratap (1978a), Santhanam and Perumal (2003), and Santhanam *et al.* (2012). The zooplankton population density was comparatively more when organic matter from the catchment areas was higher as opined by Santhanam and Perumal (2003). The least number of species recorded during the monsoon season might be due to the heavy monsoonal flood, which could not survive in very low salinity. Similar observations were made earlier by Maruthanayagam and Subramanian (1999) and Perumal *et al.* (2008). The high salinity and the more number of species observed during the same season. The minimum values of diversity was recorded during the monsoon season, being higher during other periods. Maximum and minimum species richness noticed during summer and monsoon seasons, respectively, could be interconnected with lowest and highest salinity values as reported by Mani (1992).

In the present investigation species evenness was found to be lowest in summer season due to distribution of the species in these months, and the maximum evenness values were obtained during monsoon season in all the stations which showed that the species were similarly distributed and thus not allowing a single species to be dominant over others as

reported earlier by Kumar (1993) from Vellar estuary, Karuppasamy and Perumal (2000) from Pichavaram mangrove waters, and Saravanakumar *et al.* (2007) from Gulf of Kachchh waters. The correlation matrix results between the physico-chemical and copepods were shown in Tables 2, 3, 4 and 5. The strong positive correlation were found between rainfall and pH, atmosphere temperature, phosphate, nitrate and salinity whereas strong negative correlation were found between dissolved oxygen and rainfall (Table 2). Atmospheric and surface water temperatures have been positively correlated with copepod population density, copepod diversity, copepod richness and copepod evenness (Table 4). The same trend has been observed in station 3 also (Table 5). Inorganic phosphate negatively correlated with nitrate and dissolved oxygen in station 3.

R-mode Varimax factor analyses

Station-1

Rotation mode factor analyses were plotted in Fig. 17. During the sampling period in Station 1, the R-mode Varimax factor analyses data indicated a total four factors responsible for 87.68 % of the variance (Table 6). Factor 1 accounted for 40 % of the total variance due to strong positive loadings of salinity, atmospheric temperature and surface water temperature, nitrite have moderate positive loading of chlorophyll 'a', population density, species diversity and species richness. In this factor, the nutrients significantly correlated (Table. 6) with pH, dissolved oxygen, phosphate, nitrate, silicate, species evenness. Previous studies confirmed that the nutrients and the main factors determining the concentrations of plankton community in coastal waters (Dinesh Kumar *et. al.*, 2017). In this factor termed as plankton productivity factor. Factor 2 explains 66% of the total variance and associated with moderate positive loading of rainfall, phosphate, nitrate and ammonia, weak positive loading of salinity and silicate strong negative loading of dissolved oxygen,

moderate negative loading of nitrite and silicate. In this case, rainfall positively correlates with all the nutrients except ammonia (Table 6). Similarly correlates with population density and negatively correlates with phosphate and nitrite. While the relationship between rainfall and water transparency is significant, indicating that the contribution of rainfall to water transparency is associated with river or water influx by rainfall. On other hand, there was a significant positive correlation of water transparency with chlorophyll 'a' and population density. In this factor called as rainfall factor. Factor 3 explains 78% of total variance and was associated with strong positive loadings of pH, ammonia and silicate. Hence the case, pH, ammonia and silicate shows strong positive affinity this is called as alkalinity factor. Factor 4 explains 88% total variance and is associated with positive loading of ammonia. This termed as diversity factor.

Station-2.

Factor 1 explains 36% of total variance and strongly loaded positively with species richness, surface water temperature, population density and salinity, moderately loaded positively with atmospheric temperature, nitrite, and chlorophyll 'a', strong positive of dissolved oxygen, silicate and ammonia, and weak negative loadings of rainfall. This factor termed as environmental factor. Factor 2 explains 65% of total variance and is strong positive relationship with pH, dissolved oxygen moderately loaded positively with pH, strong negatively rainfall, phosphate, and nitrate. Factor 3 explains 76% of the total variance and indicates strong positive loading of nitrate and silicate. This factor called as nutrient factor.

Station-3

In the case of Station 3, totally 4 factors explain about 77.6% of the total variance (Table.6). Factor 1 explains 65% of total variance and is associate with strong positive

loadings (Table. 6) of atmospheric temperature, surface water temperature, nitrite, population density, species diversity, species richness, silicate and chlorophyll 'a'. This factor called productivity factor. Factor 2 is responsible for 53% of total variance and shows a strong positive loading of salinity, dissolved oxygen and chlorophyll 'a' and strong negative loading (Table 6) of nitrate, rainfall, phosphate and nitrite. This factor called as Riverine factor. Factor 3 explains 74% of total variance and shows strong positive loading of silicate, species evenness, moderate negative loading of phosphate. This factor called as nutrient factor. Factor 4 explains 82% of total variance strong positive loading of pH and ammonia. This factor termed as alkalinity factor.

Chapter III

6. Morphological and Molecular Identification of Marine Copepod *Oithona dissimilis*

Introduction

Tiny copepods constitute the main component of primary feeding diet of many pelagic carnivorous fishes and they are found to be more abundant than any other type of live feed organisms. (Ajiboye *et al.*, 2011; Shansudin *et al.*, 1997; Spinelli *et al.*, 2011). The crustacean genus, *Oithona* is represented by a small size pelagic copepods that are distributed all over the World's Oceans and seas particularly in Tropical and Polar seas (Nielsen and Andersen, 2002; Paffenhöfer, 1993; Saiz *et al.*, 2003 Chew and Chong, 2011; Chew *et al.*, 2015; Dvoretsky and Dvoretsky, 2015; Wang *et al.*, 2015). In the tropical region, copepods are dominantly distributed in neritic areas (Rezai *et al.*, 2004; Chew and Chong, 2011). Being a microscopic form, they play an important role in regeneration and exporting of nutrients (McKinnon and Ayukai, 1996, Zamora-Terol *et al.*, 2014a). The common copepod, *Oithona* plays a crucial linking role in marine food chain and it feeds on phytoplankton and microbial components. The copepods are being preyed upon by larger zooplankton and several pelagic ichthyoplankton (Castro *et al.*, 2010; Spinelli *et al.*, 2011; Van Noord *et al.*, 2013). In spite of their richness and important ecological role in the function of tropical marine diversity, only very little information is available for *Oithona* group especially on their biology and ecology.

The copepod, *O. dissimilis* Lindberg, 1940 is widely distributed in estuaries (South East Asian continent and Islands of the tropical and subtropical West Pacific) and it is a dominant member of the zooplankton community (Ferrari, 1977; Oka and Saisho, 1994; Lo

et al., 2004; Saitoh *et al.*, 2011). However, accurate identification of the species of the genus, *Oithona* remains chaotic due to their small body size and subtle morphological differences among species. Of late, molecular identification has been applied systematically on copepods for accurate taxonomic identification of species. Very little information is available for cyclopoid copepods, especially for the species of *Oithona*. So far, studies have been concentrated on *O. similis*, *O. atlantica*, *O. nana* (Georgina *et al.*, 2012) and *Dioithona rigida* (Radhika *et al.*, 2017) and no data is available for *O. dissimilis*. Therefore, molecular approach for our species is still lacking which create taxonomic uncertainty in *Oithona* group at species level. To overcome the problem, “total evidence” approach in identifying copepod, by using both morphological and molecular evidences has to be followed and whenever possible (Mcmanus and Katz, 2009). In this study, I have used the morphological characters besides molecular markers (18S rDNA gene) for the species identification of copepod collected from Nagroe, Nagapattinam coastal water to compare its sequence similarity, phylogenetic and genetic divergences with the available sequences of the related species from NCBI data base.

Materials and Methods

Sample collection and identification

Copepod samples were collected from the Nagapattinam coastal waters (Fig. 1) (Lat. 10.83° 03' N; Long. 79.86° 47' E) during early morning, using plankton net (0.35m mouth diameter) made up of bolting silk cloth (No. 10, mesh size 158-µm) by its horizontal towing for about 20 minutes. The collected samples were immediately transported to the laboratory and vigorously aerated using battery aerator. The zooplankton sample was thoroughly rinsed to reduce contamination of another zoo and meroplankton. The zooplankton sample was initially screened to isolate the size fractions containing predominantly adult copepods and later-stage copepods. Then, the rotifer, nauplii of copepod and barnacles were removed by

rinsing the samples, through zooplankton washer fitted with 190 µm mesh size. This was achieved by a first coarse screening through a 500-µm mesh to eliminate the fish and prawn larvae. Specimens of the target cyclopoid copepod *Oithona* were isolated, separated and their morphological characters were observed by using standard keys (Davis, 1955; Kasturirangan, 1963; Perumal *et al.*, 1998 and Santhanam and Perumal, 2008). The isolated copepods were observed under a stereo-phase contrast microscope and their images were taken with a digital still camera. After morphological identification, the separated copepods were preserved in 5% formalin for further morphological taxonomic study and 95% ethanol preservation for molecular analysis.

Genomic DNA isolation, PCR analysis and DNA sequencing

Copepod genomic DNA isolation

Sample preparation for DNA isolation

The copepods were prepared for molecular analysis (both PCR and sequencing) by rehydrating individual copepods in 0.5 ml of distilled water in a micro-centrifuge tube for 24 h. In some cases, individual copepods were boiled in distilled water for 10 min to evaporate the alcohol before molecular analysis.

Isolation of DNA from copepods (Spin-Column Protocol as per manufacture instructions)

- ✓ Individual copepods were cut into two half and placed in 1.5 ml microcentrifuge tube, to this 180 µl ATL buffer and 20 µl of proteinase K were added.
- ✓ The microcentrifuge tube was vortexes and incubated at 56°C for 2h. After incubation, 200 µl of AL Buffer and 200 µl 100% ethanol were added. The mixer was vortexes again. After vortex, the mixture was placed in DNeasy Mini spin column with 2 ml collection tube.
- ✓ The copepod samples were centrifuged at 6000 x g (8000 rpm) for 1 min using cooling centrifuge (D LAB, D30 24R, China) and discarded the flow-through and

the collection tube.

- ✓ DNeasy Mini spin column was placed in new 2 ml collection tube and 500 µl buffer AW1 was added. The column was centrifuged at 8000 x g rpm. Flow- through and collection tube was discarded.
- ✓ DNeasy Mini spin column was placed in new 2 ml collection tube and 500 µl Buffer AW2 was added and centrifuged at 20,000 x g (14,000 rpm) for 3 min. Flow throw of the collection tube was discarded out.
- ✓ The excess flow was allowed through the collection tube, DNeasy Mini spin column was once again placed in old collection tube (step 5) and column was centrifuged at 14,000 rpm for 1 min. Flow throw and collection tube was discarded out. Then, the DNeasy membrane was allowed to dry for one minute at room temperature.
- ✓ The spin column was placed in a clean 2 ml micro centrifuge tube, to this, 200 µl AE buffer was added. The spin column was again incubated at room temperature for 1 min, and then centrifuged at 6000 x g (8000 rpm) for 1 min.
- ✓ The flow through contained the DNA was collected. Collected DNA was stored in 4°C until the further analysis.

Autoclaved de ionized water	13.5 µl
*10X <i>Taq</i> buffer	1.5 µl
Forward primer 1 µm/µl	1.0 µl
Reverse primer 1 µm/µl	1.0 µl
dNTPs 10 mm/µl	1.0 µl
<i>Taq</i> polymerase 1.5 u/µl	1.0 µl
DNA Template	1.0 µl (~50 ng)
Total Volume for one reaction	20 µl

Table 7. PCR reaction mix was used for molecular analysis

Cytochrome c oxidase subunit I (Folmer <i>et al.</i> , 1994)	
LCO1490:	5'-GGTCAACAAATCATAAAGATATTGG-3'
HC02198:	5'-TAAACTTCAGGGTGACCAAAAAATCA-3'

Table 8. The primers used for copepod molecular analysis

Steps	Action	Time (min)
Initial denaturation	94°C	5
Denaturation	94 °C	1
Annealing	52 °C	1
Extension	72 °C	1
Gone to	Step 2	30 times
Final extension	72 °C	3
Held	4 °C	

Table 9. The conditions maintained in PCR program

Agarose gel electrophoresis

- ✓ 1 g of agarose was dissolved in 100 ml of 1X Tris Acetate Ethylenediamine tetraacetic acid (TAE) buffer.
- ✓ Agarose mixture was heated in the microwave oven for 60 sec, 90 sec interval with constant shaking.
- ✓ The mixture was swirled and was made sure that agarose has melted without any formation of lumps or particles.
- ✓ Agarose was allowed to cool for several minutes and 2 µl (10mg/ml) of Ethidium bromide was added. Agarose solution was poured into a sealed gel tray without any air bubbles the wells were prepared with desired comb.
- ✓ After the gel had completely hardened, the comb was carefully removed and the gel was immersed in 1X TAB running buffer.

- ✓ PCR amplified products were loaded with 6X gel loading dye into wells, 100 bp DNA ladder was also added for reference.
- ✓ Electrical leads were connected to the electrophoresis chamber and the gel was run with a constant Volt of 90 V for 30 min. The gel was carefully removed and viewed with Gel documentation system with UV Filter (BioRad, 1708195, USA) and the amplified products of the PCR were visualized and the images were captured.

Bioinformatics 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 and similarity sequences were retrieved for phylogenetic analysis. A multiple alignment of all similarity sequences was done by Clustal W 2.1 for the determination of the levels of differentiation between genera and species. Phylogeny analysis was carried out by neighbor joining (NJ) (Saitou and Nei, 1987) search with Kimura 2-parameter as model which was using MEGA version 4.0.2. The tree was bootstrapped using 1000 sub replicates. Similarly, pair-wise nucleotide distances were estimated with the Kimura 2-parameter among the obtained partial 18S rRNA sequence and out groups were calculated by using MEGA Ver. 4.0.2 (Tamura *et al.*, 2007).

Result

Morphological identification *O. dissimilis*

Taxonomical Classification of *O. dissimilis*

Phylum	:	Arthropoda
Class	:	Hexanauplia
Subclass	:	Copepoda
Order	:	Cyclopoida
Family	:	Oithonidae
Genus	:	<i>Oithona</i>
Species	:	<i>O. dissimilis</i>

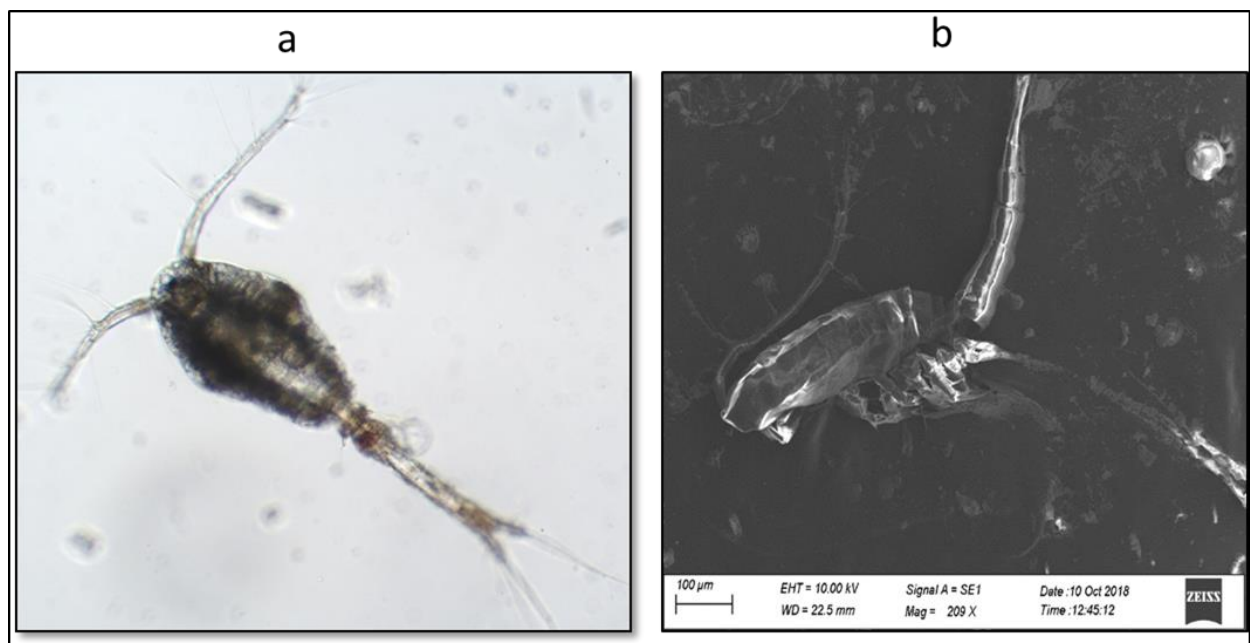
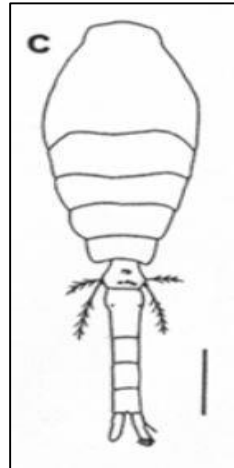


Fig. 19. Inverted phase-contrast microscopic (a) and scanning electron microscopic (b) images of *O. dissimilis*

Taxonomic Key Characters:

Female: Metasome segments 1-4 each with a pair of dorsal sensory hairs (segment 2 with 2 pairs). Excluding terminal spine, exopod of P1-P4 with 1-1-3, 1-1-3, 1-1-3, 1-1-2 external spines, respectively; 1-1-4, 1-1-5, 1-1-5, 1-1-5 internal setae, respectively. Endopod P1-P4

with 0-0-1, 0-0-1, 0-0-1, 0-0-1 external setae, respectively and 1-1-5, 1-2-5, 1-2-5, 1-2-4 internal setae respectively. 5th thoracic segment without hairs on the posterior margin. Caudal rami longer than the 5th thoracic segment; Proportions of the urosomal segments 12, 33, 14, 14, 13. P5 bears a fine seta which is directed dorsally, and 1 terminal seta. No ciliation found on either of these P5 setae. The terminal seta reaches almost the end of abdominal segment 1-2.

Male: A1 twice geniculated; the proximal geniculation is surrounded by a sheath; distal geniculation with a notch in the segment. Caudal rami shorter than in female; Si very short and can only be seen. Proportions of the urosomal segments caudal rami: 19: 19: 16: 13: 10:11: 13. Prosome laterally with a very complex group of integumental organs in an area comprising posterior ventral part of cephalosome and posterior extension or flap of cephalosome overlapping the following segment.

Molecular characterization of COI gene of *O. dissimilis*

Blast

The dataset was prepared for our target species *O. dissimilis* Contig-PS11 based upon similarity search. We selected out the species based on the identity (>79%) and above 98% of query coverage (Table 10).

Phylogenetic tree

Estimation of inter-and intra-specific phylogeny

The evolutionary history of COI gene of *O. dissimilis* PS-11 was inferred through the Neighbour-Joining method of analysis. The optimal tree with the sum of branch length = 4.48590762 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary

distances used to infer the phylogenetic tree. Here I have constructed the phylogenetic tree of both inter- and intra-specific organisms. The inter-specific phylogeny shows that the COI gene of *O. dissimilis* PS-11 is diverged form the strains of OdS-CO1-01, OdS-CO1-02, and OdS-CO1-03; OdS-CO1-04 and thus the present has been identified as *O. dissimilis* and the OdJ-CO1-01 act as an ancestor for our target species (Fig 19). The overall mean distance was found in the range of 1.782 and it surely indicates that, *O. dissimilis* PS-11 is involved for positive evolution of Darwinian test for inter-specific phylogeny level. Whereas the intra-specific phylogeny reveals that, the tree was classified into two major clade and four sister clades. The first clade consists of four families' viz., *Paracalanidae*, *Clausocalanidae*, *Centropagidae* and *Pontellidae* that are grouped with each other. Whereas the second clades consist of Oithonidae family as shown in Fig 19. The overall mean distance was found in the range of 0.150 and it surely indicates that, *O. dissimilis* PS-11 is involved for neutral evolution of Darwinian test and no changes has been found to occur during the evolutionary process of inter-specific phylogeny.

Estimation of pair-wise genetic diversity

The evolutionary distances were computed by following the Maximum Composite Likelihood method that in the units of the number of base substitutions per site. The analysis involved 6 nucleotide sequences. All positions containing gaps and missing data were eliminated. The genetic diversity of inter-specific phylogeny shows that *O. dissimilis* OdJ-CO1-01 is highly diverged one when compared to the other strains of *O. dissimilis* and its occurrence range is between 1.031-1.137 (Table 11). Whereas, the intra-specific pair-wise genetic diversity shows that neutral evolution will takes place and its diversity range was 0.0-0.274 (Table 12). This statistical pair-wise genetic diversity data shows that our study provided a strong conclusion.

Accession	Organism	Haplotype	Query cover (%)	E-value	Identity (%)
AB604163.1	<i>Oithona dissimilis</i>	OdS-CO1-03	98	3.00E-144	81
AB604164.1	<i>Oithona dissimilis</i>	OdS-CO1-04	98	9.00E-144	81
AB604161.1	<i>Oithona dissimilis</i>	OdS-CO1-01	98	4.00E-142	81
AB604162.1	<i>Oithona dissimilis</i>	OdS-CO1-02	98	5.00E-141	81
AB604165.1	<i>Oithona dissimilis</i>	OdJ-CO1-01	98	4.00E-117	79

Table 10: Dataset preparation of cytochrome c oxidase I of *O. dissimilis* PS-11 and its phylogenetic similarity using NCBI-BLAST

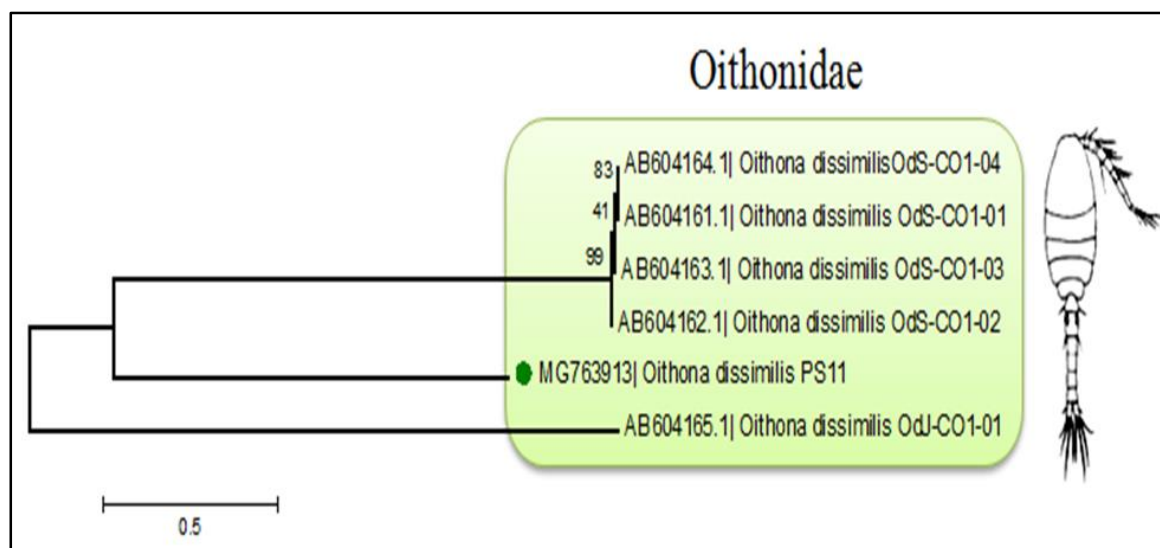


Fig. 20. Construction of inter-specific phylogenetic tree of COI gene of *O. dissimilis* PS-11 from its closely related sequences obtained from MEGA 7.0. Green colour bullet differentiates our target sequences

S. No.	Accession	Organism	Haplotype	1	2	3	4	5
1	MG763913	<i>Oithona dissimilis</i>	-					
2	AB604163.1	<i>Oithona dissimilis</i>	OdS-CO1-03	0.854				
3	AB604164.1	<i>Oithona dissimilis</i>	OdS-CO1-04	0.867	0.006			
4	AB604161.1	<i>Oithona dissimilis</i>	OdS-CO1-01	0.867	0.007	0.002		
5	AB604162.1	<i>Oithona dissimilis</i>	OdS-CO1-02	0.87	0.008	0.006	0.007	
6	AB604165.1	<i>Oithona dissimilis</i>	OdJ-CO1-01	1.031	1.137	1.131	1.131	1.116

Table 11: Estimation of inter-specific pair-wise genetic distance of COI gene of *O. dissimilis* PS-11 from its phylogenetic neighbours obtained from MEGA 7.0

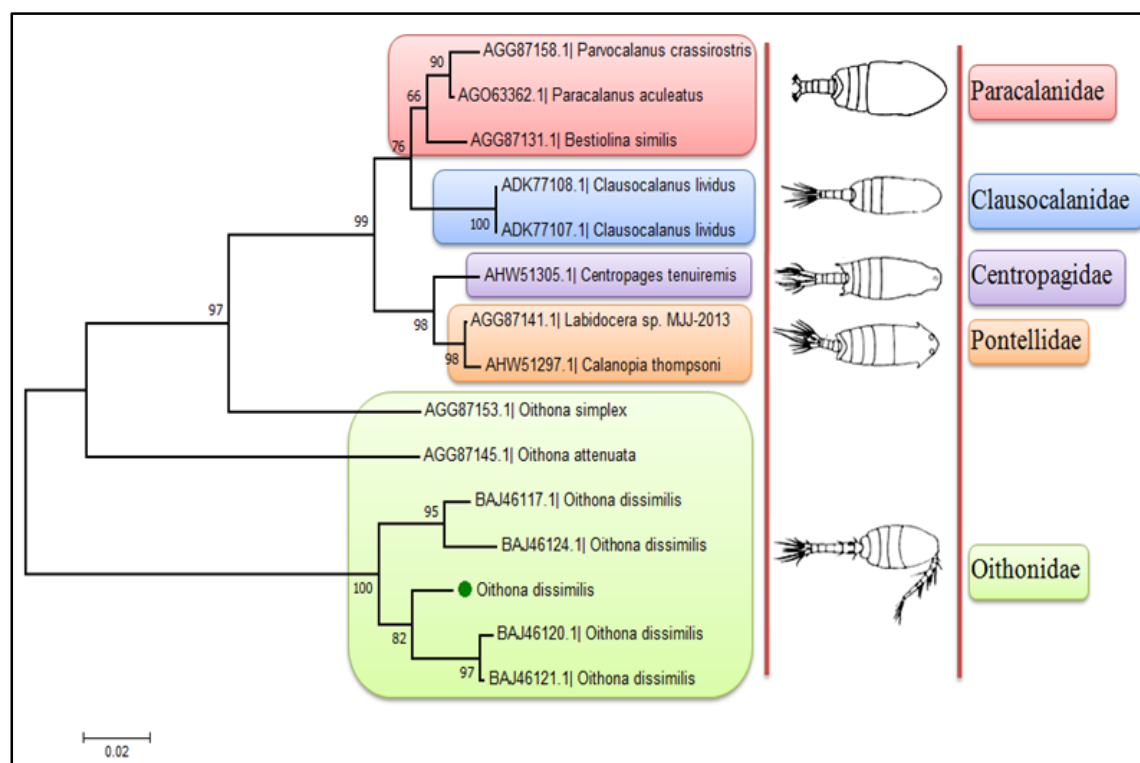


Fig. 21. Construction of inter-specific protein based phylogenetic tree of COI gene of *O. dissimilis* PS-11 from its closely related sequences obtained from MEGA 7.0. Green colour bullet differentiates our target sequences

S. No.	Organism	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	<i>Oithona dissimilis</i>																	
2	<i>Oithona dissimilis</i>	0.032																
3	<i>Oithona dissimilis</i>	0.037	0.005															
4	<i>Oithona dissimilis</i>	0.046	0.051	0.056														
5	<i>Oithona dissimilis</i>	0.065	0.065	0.07	0.023													
6	<i>Parvocalanus crassirostris</i>	0.251	0.268	0.262	0.262	0.274												
7	<i>Paracalanus acueatus</i>	0.251	0.268	0.262	0.262	0.274	0.009											
8	<i>Labidocera sp.</i>	0.268	0.28	0.274	0.268	0.28	0.061	0.051										
9	<i>Calanopia Thompson</i>	0.274	0.286	0.28	0.274	0.274	0.065	0.056	0.005									
10	<i>Oithona simplex</i>	0.274	0.28	0.28	0.268	0.251	0.125	0.125	0.125	0.125								
11	<i>Centropages tenuiremis</i>	0.262	0.274	0.268	0.262	0.274	0.065	0.056	0.023	0.028	0.135							
12	<i>Clausocalanus lividus</i>	0.251	0.268	0.262	0.268	0.28	0.046	0.037	0.061	0.065	0.146	0.061						
13	<i>Bestiolina similis</i>	0.251	0.274	0.268	0.268	0.28	0.028	0.018	0.056	0.061	0.125	0.061	0.042					
14	<i>Labidocera sp.</i>	0.268	0.28	0.274	0.268	0.28	0.061	0.051	0.0	0.005	0.125	0.023	0.061	0.056				
15	<i>Bestiolina similis</i>	0.251	0.274	0.268	0.268	0.28	0.28	0.018	0.056	0.061	0.125	0.061	0.042	0.0	0.056			
16	<i>Clausocalanus lividus</i>	0.251	0.268	0.262	0.268	0.28	0.046	0.037	0.061	0.065	0.146	0.061	0.0	0.042	0.061	0.042		
17	<i>Centropage abdominalis</i>	0.268	0.268	0.262	0.268	0.28	0.065	0.056	0.018	0.023	0.125	0.042	0.046	0.065	0.018	0.065	0.046	

Table 12: Estimation of inter-specific pair-wise genetic distance of COI gene of *O. dissimilis* PS-11 from its phylogenetic neighbours obtained from MEGA 7.0.

Discussion

The cyclopoid copepod, *Oithona* is highly abundant, ecologically important and is widely distributed throughout the marine environs. The routine identification of the copepods of this genus remains a challenge due to their small size and subtle morphological diagnostic traits (Radhika *et al.*, 2017). The copepod, identified in my study was very well characterized by the presence of the prominent features of *O. dissimilis* on the basis of antenna (A1) which is shorter and both are geniculate in male and descriptive features confirmed that the males are usually smaller than females, urosome was 6 segmented in male and 5 segmented in female (Inshida 1985). In order to differentiate species within the genus, the prominent characters being conventionally followed are based on the arrangement of setae and spines on the exopod of swimming legs 1-4 (Radhika *et al.*, 2017). Presently, the setae and spines of P1-P4 arranged in our specimen were consistent with the keys provided by Wellershaus (1969) and hence that our copepod was identified as *O. dissimilis*.

Bucklin *et al.* (2003) have confirmed that mt COI sequence variation has been proved to be a successful marker in molecular systematic and phylogenetic evolution in copepods. The application of COI gene for the DNA barcodes has proved to be a useful marker particularly for copepods (Hill *et al.*, 2001; Bucklin *et al.*, 2003). This gene has also been useful to distinguish the closely related genera for species identification (Paine *et al.*, 2007). Accordingly, we have examined the mt COI gene used for the identification and discrimination of *O. dissimilis* in relation to phylogenetic and evolution of copepods. Molecular phylogenetic analysis based upon mt COI clearly revealed that our strain is distinct from the other related copepods.

Presently, I have sequenced mt COI gene and compared its molecular features with the already publically available data on different species of different families available from NCBI. The mt COI gene of the species collected from Nagore, Nagapattinam coastal waters was subjected to BLAST and found that intra species (*Oithona dissimilis*) was the most closely related species with 81% similarity (98% - Query coverage). Thus, our blast similarity was reliable with finding of Soh *et al.* (2012) who implied that COI gene is an appropriate marker for the identification of species because it has enough diversity to address intra and inter specific phylogenetic relationship for invertebrates (Soh *et al.* 2012). The phylogenetic relationships among *Oithona* sequences from NCBI with our selected samples using mt COI gene was well resolved. COI gene would be an appropriate biomarker for species discrimination as it has been widely employed to study the population genetics and evolution (Shao and Barker, 2007). It is the most conservative protein-coding gene found in the mitochondrial genomes of animals (Brown, 1985). It was clearly found in our study that the overall mean distance for intra specific phylogeny was found to occur in the range of 1.782 indicating that *O. dissimilis* PS-11 was involved for positive evolution of Darwinian test for intra-specific phylogeny level. For inter specific phylogeny, the overall mean distance has occurred in the range of 0.150 indicating that, *O. dissimilis* PS-11 was involved for Neutral evolution of Darwinian test and no changes have occurred during the evolutionary process of inter-specific phylogeny.

A higher level of genetic distance was found among intra species within our strain. The occurrence of higher level of genetic distance in our strain with intra and inter species level might be due to the presence of cryptic or new species or sub species and so on. Although the copepods have been shown to reveal higher levels of genetic divergence, but sometimes the observed morphological conservatism might not follow the same level of genetic divergence. This might be due to the fact that reproductive isolation has not been

uncoupled from morphological divergence (Goetze 2003). COI gene sequence analysis have clearly showed that the within-species variation occurred due to the presence of cryptic or sibling species within many crustaceans but also identified such levels of speciation in other eukaryotes (Waugh, 2007). So, detailed morphological, molecular and behavioral studies of a population of closely related organisms of *Oithona* species must be focused in future. In the present study, *O. dissimilis* was able to survive, produce more nauplii and population density at a temperature range of 28°C - 32°C as reported earlier researchers for other copepods (Rajthilak *et al.*, 2014; Peter and downing 1984; kaviyarasan *et al.*, 2019; Santhanam and Perumal, 2012).

Chapter IV

7. Optimization of Culture Conditions for Marine Copepod *Oithona dissimilis*

Introduction

Generally, copepods are primary food source for many fish and crustaceans. Utilizing copepod as a live feed would enhance the larval survival, increase the growth rate, as they possess high HUFA-content and has broad range of body size. Now a days, Artemia and Rotifer are being applied as live feed for larval rearing in aquaculture practices (Santhanam and Perumal, 2011). But they are insufficient in terms of essential nutrients that are required for the good growth and overall development of larvae. Therefore, much attention is required on the mass culture of copepods so as to utilize the cultured copepods by the larvae. Research laboratories in various parts of the world have been working on culturing copepods so as to produce potential live feed suitable for aquaculture industry (Santhanam and Perumal, 2011). However, due to inconsistency in production due to inefficient culture procedure, the copepods are not became popularized among aquafarmers. Proper standardization of growth and reproduction of cyclopoid copepods in the field is not easy.

However, conducting laboratory experiment on the culture of copepods by adopting similar natural environmental parameters is the best way to determine the optimum requirement for the production, growth, and reproductive parameters. Such experiment is important for the successful culture of copepods that could be used as live feed in the aquaculture industry (James and Al-Khars, 1986; Hernandez Molejon and Alvarez-Lajonchere, 2003). In relation to the mounting importance on copepods as live feed in aquaculture, it is essential to understand the basic knowledge on its biology. The quality and

quantity of the diet, salinity and temperature are probably the dominant factors which controls the productivity of copepod. The effects of different microalgal diets on egg producing capacity (Kleppel *et al.*, 1998; Koski and Kuosa, 1999; Payne and Rippingale, 2000; Santhanam and Perumal, 2012a), egg hatching success, mortality and development (Knuckey *et al.*, 2005; Leandro *et al.*, 2006) have been documented for several calanoid species. Some studies have investigated the response of paracalanoid copepods to different food sources, salinity regimes and temperature (McKinnon *et al.*, 2003; VanderLugt and Lenz, 2008). Productivity of copepods in intensive culture is directly depended to female egg production, and also a measure of the net production rate of adult females (Shin *et al.*, 2003). However, subsequent egg hatching rate, naupliar and copepodite survival and development rates, all impact the productivity of copepod cultures (Milione and Zeng, 2007; Santhanam *et al.*, 2013). Due to increasing importance of copepods as live food for tropical aquaculture, information on mass culture of these organisms is critical to support the growing industry. The aim of this study is to provide information on the effects of temperature, pH, salinity, light, feed and feed concentration on the survival, nauplii production, population and development of a tropical cyclopoid copepod, *O. dissimilis* as this organism has the potential as live food for mariculture use.

Materials and Methods

Microalgal culture

The marine microalgae such as, *Isochrysis galbana* (ISO), *Chlorella marina* (CHL), *Picochlorum maculatum* (PICO), *Nannochloropsis oculata* (NAN) and *Amphora subtropica* (AMS) were cultured in micro algae culture facility of Marine Planktonology and Aquaculture Laboratory, Department of Marine Science, Bharathidasan University, Tiruchirappalli, India. They were grown at the temperature range of 23°-25°, at the salinity of 30 PSU and with a light intensity of 45-60 mmol photons/m²/sec with a photoperiod of 12

hrs light and 12 hrs dark. All the microalgal strains were cultured using Conway's medium (Walne, 1974). The seawater used for the culture was filtered using 1 μ m filter bag and sterilized using autoclave. The containers used for the algal culture were thoroughly washed and sterilized properly before use. The microalgae harvested at the exponential phase and fed to copepods.



Plate 7. Microalgae culture facility

Maintenance of copepod stock culture

To maintain the mother culture, a known number of both male and female (50 individuals) of *O. dissimilis* were isolated and stocked in 1 litre beaker containing filtered seawater. The copepods were fed with mixed micro algae (equal amount of ISO, CHL, PICO, NAN and AMS) at the concentration of 30,000 cells/ml for every day. The salinity and temperature of the culture medium were adjusted to 26 PSU and 28°-30° respectively. The fecal pellets and debris were siphoned out daily and the expelled culture water was replaced with fresh filtered seawater.

The water quality parameters were maintained by regular monitoring of pH, salinity and temperature. The generation time of *O. dissimilis* under optimal conditions was about 10-12 days by having 6 nauplii and 6 copepodite stages including the adult. Finally, the adult gravid female copepods were used to restart the mass culture. The axenic copepod culture was maintained at Marine Planktonology & Aquaculture Laboratory under controlled conditions.



Plate 8. Copepod culture facility

Optimization Experiments

Survival Rate (SR)

The survival rate of copepod with reference to water quality and dietary conditions were studied under different temperature, light intensity, pH, salinity, diets and diets concentration for a period of 15 days. Ten numbers of healthy gravid female (*O. dissimilis*) individuals were picked up from the stock culture using stempel pipette and transferred to a 100-ml beaker containing sterile seawater (which was filtered with 1µm filter bag) with three times replication. The culture was maintained and the number of live copepods were counted at daily interval for a period of 15 days. If the dead copepods were found, they were removed from the beaker daily. The experiments were done in triplicate and extended for a total period of 15 days. The debris and fecal materials were removed by daily basis

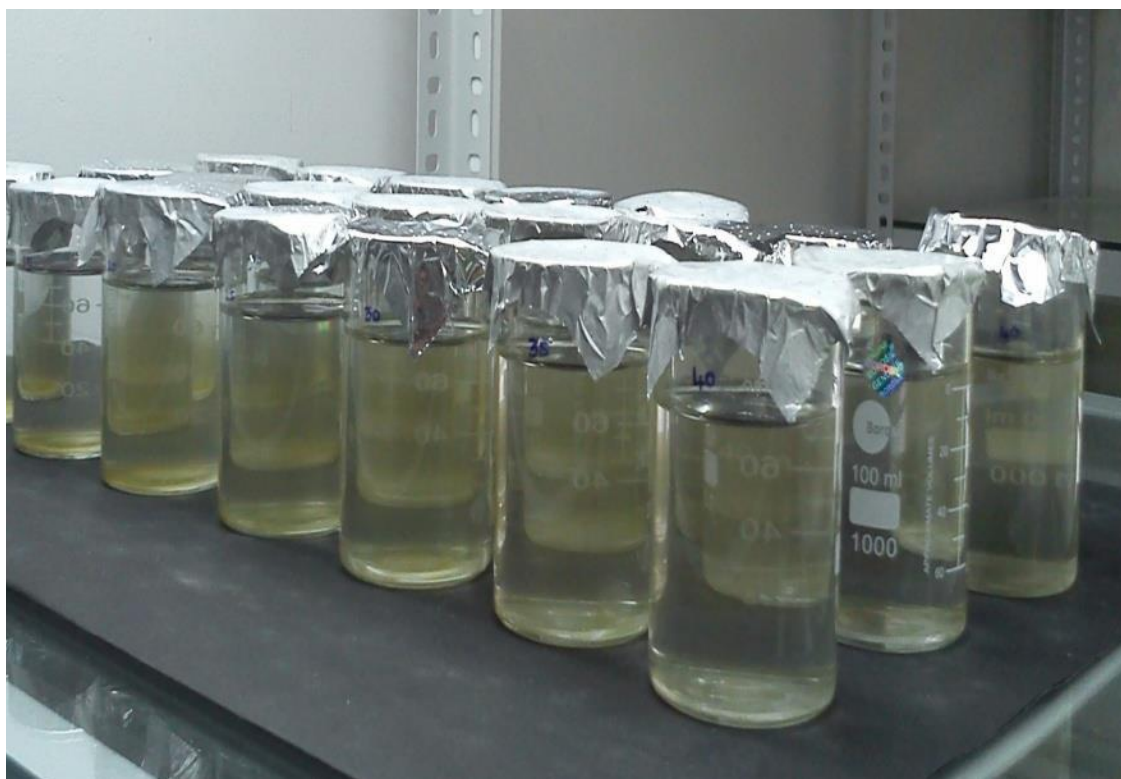


Plate 9. Experimental setup for the survival of copepod *O. dissimilis*

Nauplii Production Rate (NPR)

The nauplii producing capacity of *O. dissimilis* was assessed in relation to temperature, light intensity, pH, salinity, different diets and diets concentration. An individual female with viable egg sacs was stocked in a glass test tube containing 25 ml of filtered seawater. This set up was maintained in triplicate. The copepod was examined at regular intervals (every 1 or 2 hour) for the release of nauplii. Once the nauplii were released, the adult female was carefully removed from the test tube and the released nauplii were counted under the stereo phase-contrast microscope (Austria Micros MCX1600).

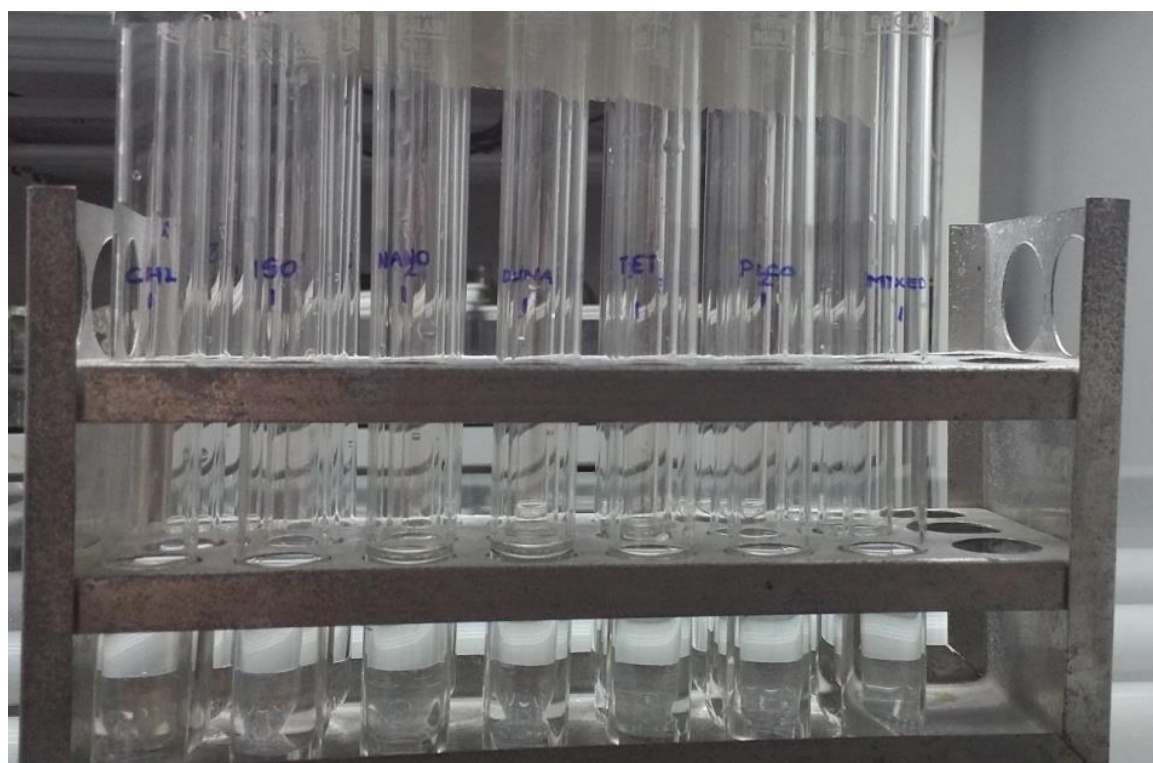


Plate 10. Experimental setup for the nauplii production rate of copepod *O. dissimilis*

Population density (PD)

To assess the copepods population density with reference to various water quality and dietary conditions, 10 adult copepods were isolated from the stock culture and inoculated in to each 500-ml beaker filled with filtered sterilized seawater. This set up was

maintained in triplicate. The population densities of *O. dissimilis* were estimated in relation to different temperatures, light intensity, pH, salinity, different diets and diets concentrations. At the end of the experimental period (15th day), the animals were harvested through 48µm mesh and fixed with 5% formalin. Different stages of copepods (nauplii, copepodites and adults) were counted under the microscope.



Plate 11. Experimental setup for population density of copepod *O. dissimilis*

Post embryonic development (PEM)

To assess the developmental period, a gravid female was stocked in a clean test tube. The copepod was checked for hatching at regular intervals. After hatching of the nauplii, the female was removed from the plate and the nauplii were observed for further development. In this experiment, the time taken for eggs to hatch nauplii (embryonic development), the development of nauplii stages (NI-NVI) and copepodite stages (CI-CVI) were noted. The generation time (egg to egg) was also recorded. Few nauplii from each treatment were cultured separately until they spawn to determine the generation time.



Plate 12. Microscopic observation on post embryonic development of *O. dissimilis*

Statistical Analysis

The obtained data on the survival rate (SR), nauplii production rate (NPR) and population density (PD) of *O. dissimilis* in relation to temperature, light intensity, pH, salinity, different diets and diets concentration were analysed by using one-way ANOVA. If significant differences ($P < 0.05$) were found, Tukey's multiple comparisons test was used to determine the specific difference among treatments. Data are presented as Mean $\pm$ SE.

Results

Optimization of Temperature

Survival rate

Different temperatures were tested on survival, nauplii production and population density. It was noticed that there was significant difference found at survival rate in percentage of copepods. There was above 50 % of survival occurred in almost all the

temperatures tested. The highest survival rate (90%) was noticed at 28° C on the final day which was followed by 32°C (70.33%), 24°C (66.6%) and 20°C (56%). whereas, the lowest survival rate (50%) was observed at 36°C. However, there was a gradual decrease in survival noticed from the beginning of the first day towards the final day at temperature 36° C (Fig. 21).

Nauplii production rate

In all the experiment performed, the temperature was found to affect the nauplii production rate. The highest NPR (21.33 nauplii/female) was noticed at 24°C which was significantly higher ($P<0.001$) than at 20°C as well as at 36°C except at 24°C and 32°C which showed considerably significant difference ($P<0.05$). The lowest NPR was observed at 20°C and 36°C with only 11.66 nauplii/female which was significantly lower ($P<0.001$) when compared to the other temperatures tested (Fig. 22).

Population density

In all the experiment conducted, the total highest population density (279.7 ind. L⁻¹) was obtained at 28°C which was significantly higher ($P<0.001$) than the rest of the temperatures tested. The lowest population density (154 ind. L⁻¹) was obtained at 20°C which was significantly lower ($P<0.001$) as compared to the other treatments. Thus, in all the experiment, the temperature significantly affect the population density at different life stages of copepod (Fig. 23).

Embryonic development

The shortest duration taken for nauplius development (N1-N6) was 3.8 days which observed in 28°C, followed by 32°C with 4 days. The copepodite to adult development (C1-C6) was shorter in 28°C with 8.7 days and longer in 20°C with 9.8 days. The shortest generation time of 14 days was observed in 28°C whereas it was longer in 36°C with 15.8 days. (Table 13)

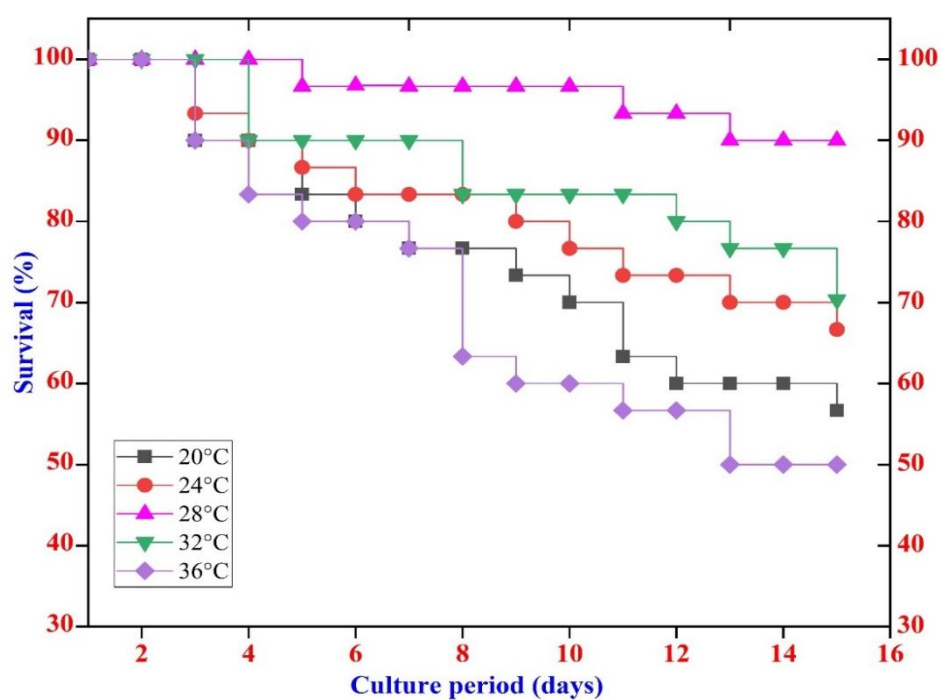


Fig. 22. Survival rate of *O. dissimilis* in respect to temperature

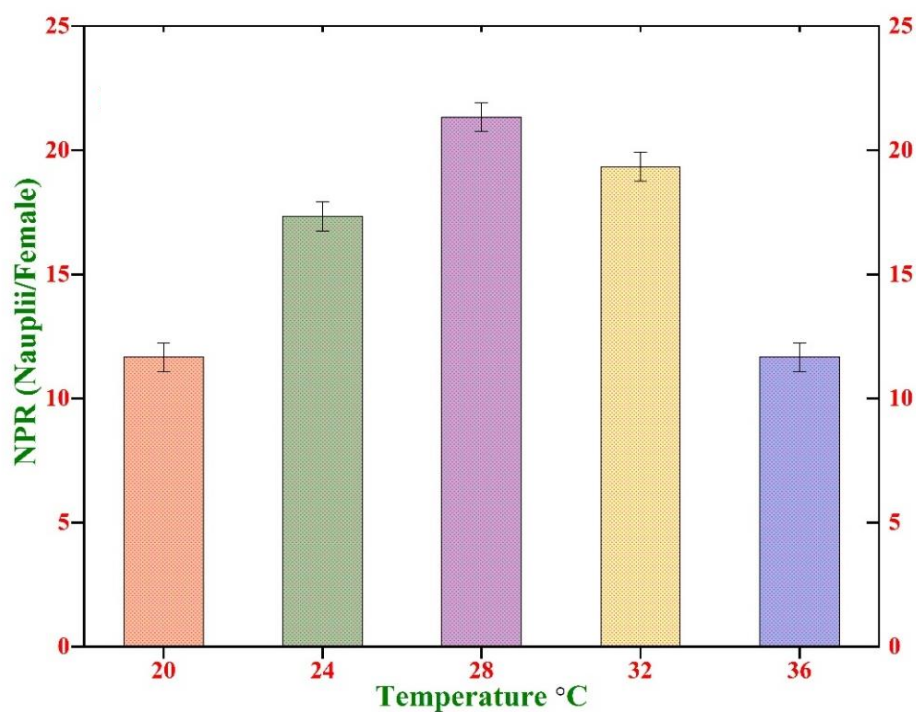


Fig. 23. Nauplii production rate of *O. dissimilis* in respect to temperature

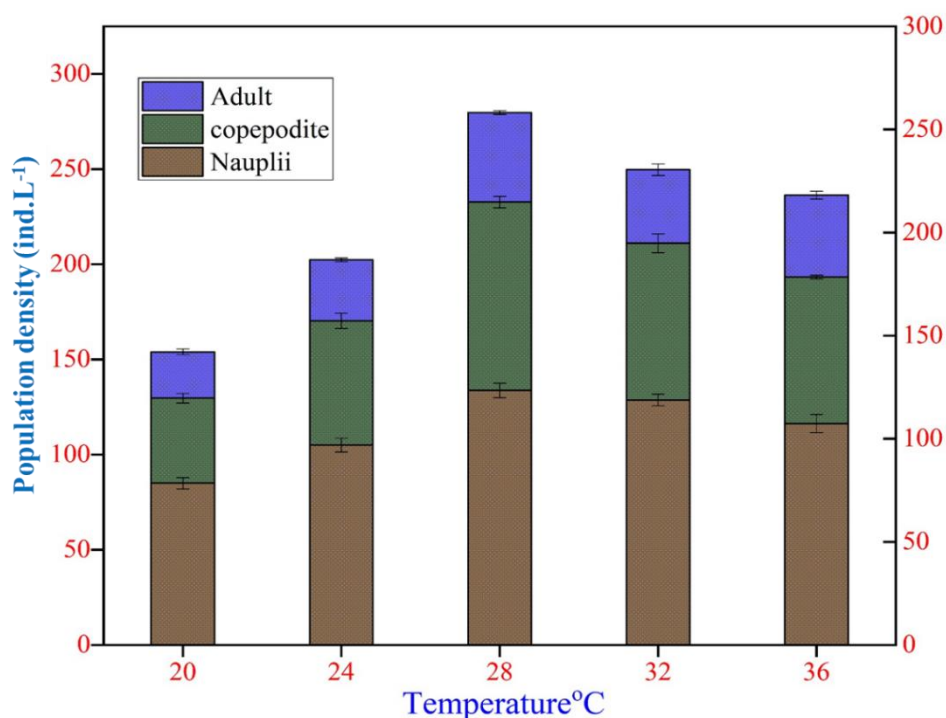


Fig. 24. Population density of *O. dissimilis* in respect to temperature

Days	Temperature (°C)				
	20	24	28	32	36
Development time (N1-N6)	4.6	4.2	3.8	4	5.2
Development time (C1-C6) (adult)	9.8	9.2	8.7	8.8	9
Total development time	14.4	13.4	12.5	12.8	14.2
Generation time	15.2	15.6	14	14.8	15.8

Note: N1-N6: Nauplii 1-Nauplii6; C1-C6: Copepodite 1-Copepodite 6.

Table 13: Effect of temperature on post embryonic development of marine copepod *O. dissimilis*

Optimization of Salinity

Survival rate

During salinity trial no significant variation was found in the survival rate of copepods. Above 50 % of survival was found in all the salinity levels tested except at 35 PSU. The highest survival rate (96.66%) was found at 25 PSU on the final day followed by

30 PSU (73.33%), 20 PSU (70%) and 15 PSU (63.3%). The lowest survival rate (40%) was obtained at 40 PSU salinity level. However, there was a gradual decline in survival from the beginning of the first day towards the final day at 40 PSU salinity (Fig. 24).

Nauplii production rate

The salinity was found to affect the nauplii production rate. The highest NPR (22 nauplii/female) was observed at 25 PSU which was significantly higher ($P < 0.001$) than at 35 PSU, 20 PSU ($P < 0.05$), 15 PSU ($P < 0.01$) and there was no significant difference ($P > 0.05$) noticed with 30 PSU salinity level. The lowest NPR was found at 35 PSU which was significantly lower ($P < 0.001$) than 25 PSU followed by 30 PSU ($P < 0.01$) except 15 PSU and 20 PSU which showed no significant difference ($P > 0.05$) respectively (Fig. 25).

Population density

The highest total population density (336 ind. L^{-1}) was obtained at 25 PSU which was significantly higher ($P < 0.001$) than the rest of the salinities tested. The lowest population density (207.6 ind. L^{-1}) was obtained at 15 PSU which was significantly lower ($P < 0.001$) when compared to other treatments except at 35 PSU which did not show any significant difference ($P > 0.05$). The same was the condition ($P > 0.05$) in population that occurred between 20 PSU and 30 PSU salinity levels. Thus, in all the trial, the salinity significantly influenced the population density at different life stages (Fig. 26).

Post Embryonic Development

The shortest duration (4.2 days) for nauplius development (N1-N6) was observed in 25 PSU, followed by 20 PSU with 4.4 days. The copepodite to adult development (C1-C6) was shorter in 25 PSU with 8.8 days and longer in 35 PSU with 9.8 days. The shortest generation time of 14.6 was observed in 25 PSU whereas it was longer in 35 PSU with 16 days. (Table 14).

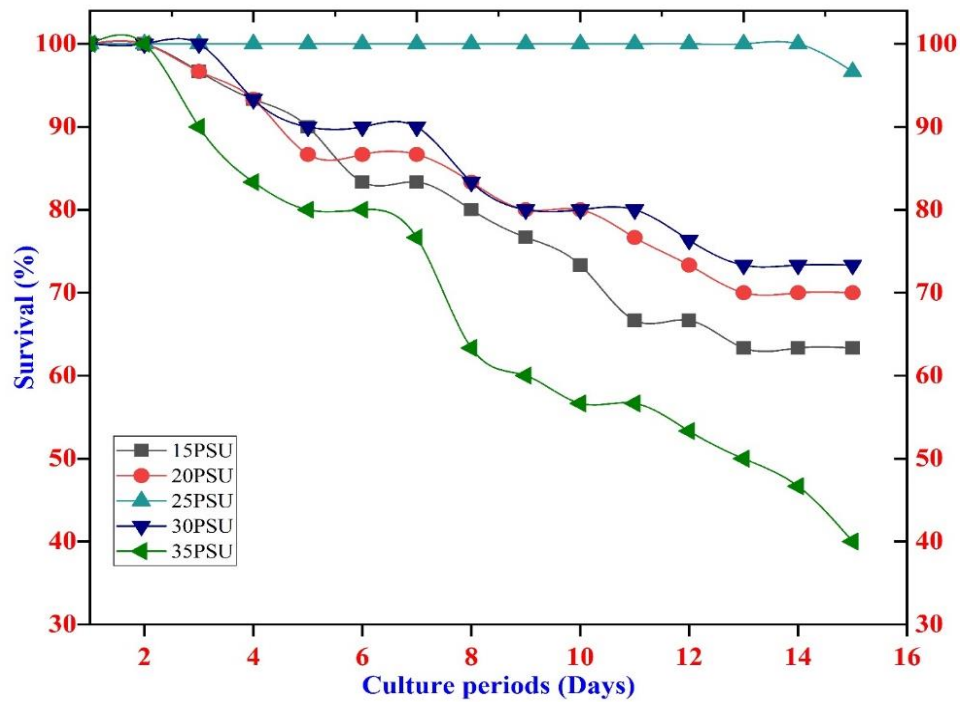


Fig. 25. Survival rate of *O. dissimilis* with reference to salinity

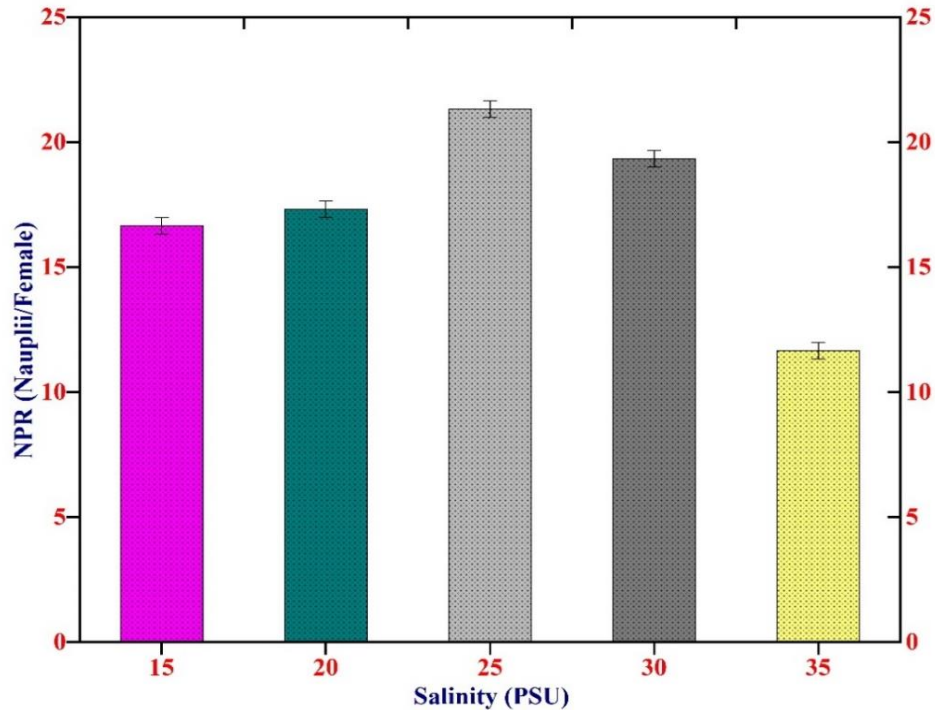


Fig. 26. Nauplii production of *O. dissimilis* with reference to salinity

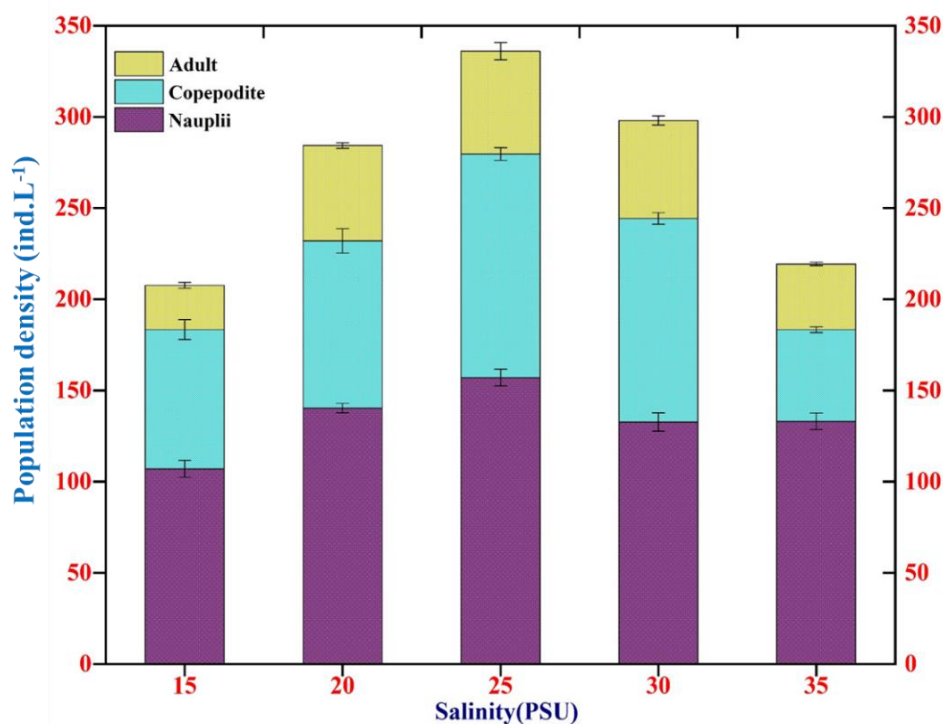


Fig. 27. Population density of *O. dissimilis* with reference to salinity

Days	Salinity (PSU)				
	15	20	25	30	35
Development time (N1-N6)	4.6	4.4	4.2	4.8	4.9
Development time (C1-C6) (adult)	9.7	9.4	8.8	9.7	9.8
Total development time	14.3	13.8	13	14.5	14.7
Generation time	15.4	15.8	14.6	14.8	16

Note: N1-N6: Nauplii 1-Nauplii6; C1-C6: Copepodite 1-Copepodite 6.

Table 14: Effect of salinity on post embryonic development of marine copepod *O. dissimilis*

Optimization of pH

Survival rate

In all pH trial conducted, above 50% survival rate was noticed except at pH 7 and pH 9. In case of pH 7 and pH 9, there was a gradual decrease in the percentage of survival from initial to final stage whereas the higher survival rate (86.6%), was noticed at pH 8

followed by pH 8.5 (83.3%). The lowest percentage in survival (40%) was found at pH 9 followed by pH 7 (43.3%) (Fig. 27).

Nauplii production rate

The pH was found to be affect the nauplii production rate in *O. dissimilis*. The highest NPR (19.66 nauplii/female) was found at pH 8 which was significantly greater ($P < 0.001$) than at pH 7 (17.33 nauplii/female) and pH 9 (9.33 nauplii/female) followed by pH 7.5. There was no significant difference ($P > 0.05$) arisen for pH 8 vs pH 8.5 and pH 7.5 vs pH 8.5 respectively. The lowest nauplii production was recorded at pH 9 (9.33 nauplii/female) which was significantly lower ($P < 0.001$) than with all other pH levels tested (Fig. 28).

Population density

In case of pH, the maximum population density (286.6 ind./L⁻¹) was found at pH 8 which was greatly significance. The minimum density (94.6 ind./L⁻¹) was observed at pH 7 which was significantly lower ($P < 0.001$) when compared to other pH levels tested except at pH 9 which showed considerable significant difference ($P < 0.05$). Thus, in all the trial, pH was significantly affected population density at different life stages. (Fig. 29).

Post Embryonic Development

The shortest duration taken for nauplius development (N1-N6) was observed in 4.2 days in 8 pH, followed by 7.5 pH with 4.5 days. The copepodite to adult development (C1-C6) was shorter in 8 pH with 9 days and longer in 9 with 9.8 days. The shortest generation time of 14.6 was observed in 8 pH whereas it was longer in 9 pH with 16 days (Table 5.).

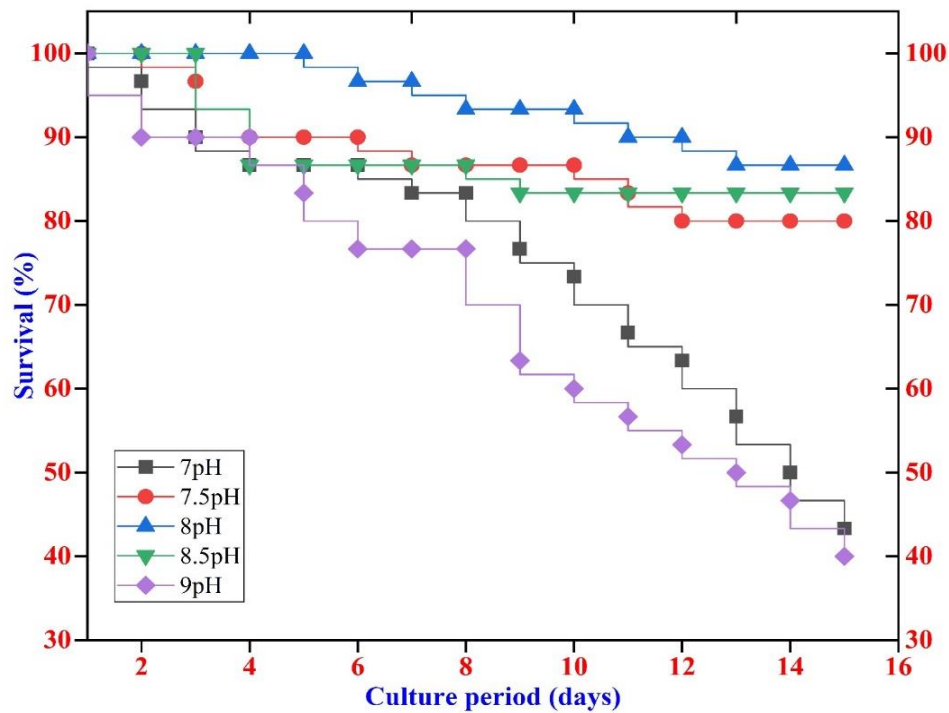


Fig. 28. Survival rate of *O. dissimilis* in respect to pH

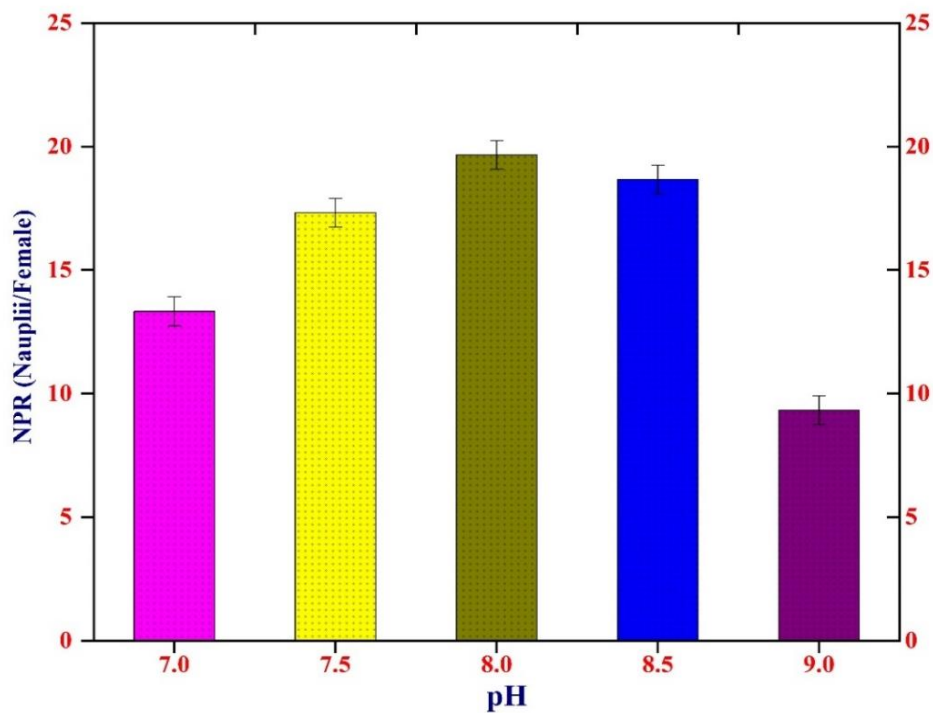


Fig. 29. Nauplii production rate of *O. dissimilis* in respect to pH

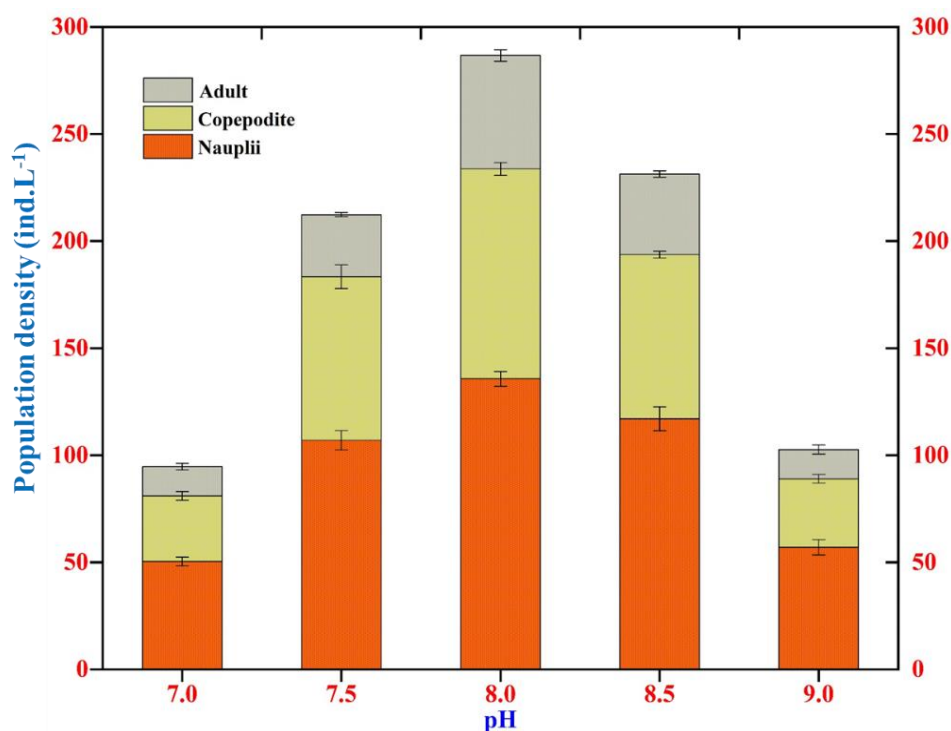


Fig. 30. Population density of *O. dissimilis* in respect to pH

Days	pH				
	7	7.5	8	8.5	9
Development time (N1-N6)	4.6	4.5	4.2	4.8	4.9
Development time (C1-C6) (adult)	9.2	9.4	9.0	9.6	9.8
Total development time	13.8	13.9	13.2	14.4	14.7
Generation time	15.4	15.8	14.6	14.8	16

Note: N1-N6: Nauplii 1-Nauplii6; C1-C6: Copepodite 1-Copepodite 6.

Table 15: Effect of pH on post embryonic development of marine copepod *O. dissimilis*

Optimization of light intensity

Survival rate

All the light intensity tested found that there was above 50% survival in almost all the intensities except at 2500 Lux where the lowest survival rate (33.33%) noticed. In high intensity (2500 Lux), there was a gradual decrease in survival rate from first day to final

day. The maximum survival (86.67%) was observed at low light intensity (500 Lux) followed by 1500 Lux (73.33%), 3000 Lux (63.33%) and 4500 Lux (53.33%) (Fig. 30).

Nauplii production rate

The production rate of nauplii was influenced by differences in light intensity. The maximum production (22.33 nauplii/female) was found at low light intensity (500 Lux) which was significantly greater ($P < 0.001$) than 1000 Lux, 1500 Lux, 2500 Lux and for 1000 Lux ($P < 0.01$) respectively. The minimum production (8.66 nauplii/female) was noticed at higher light intensity (2500 Lux) which was significantly lower ($P < 0.001$) than other intensities except 2000 Lux which showed no significant difference ($P > 0.05$). (Fig. 31).

Population density

During culture at different light intensities, the total highest population density (276 ind. L^{-1}) was obtained at 500 Lux which was significantly higher ($P < 0.001$) except 1000 Lux which did not show any significant difference ($P > 0.05$). The lowest population density (180 ind. L^{-1}) was obtained at 2500 Lux which was significantly lower ($P < 0.001$) as compared to the other intensities tested except at 1000 Lux ($P < 0.01$) and 2500 Lux ($P < 0.05$). There was no significant difference noticed in population density under 1500 and 2000 Lux. Thus, in all the trial, light intensity significantly affected population density at different life stages (Fig. 32).

Post Embryonic Development

The shortest duration (4.2 days) for nauplius development (N1-N6) was observed in 500 lux, followed by 1000 lux with 4.4 days. The copepodite to adult development (C1-C6) was shorter in 500 Lux with 9 days and longer in 2500 lux with 9.9 days. The shortest generation time of 15.2 was observed in 500 lux whereas it was longer in 2500 with 16.2 days (Table 16).

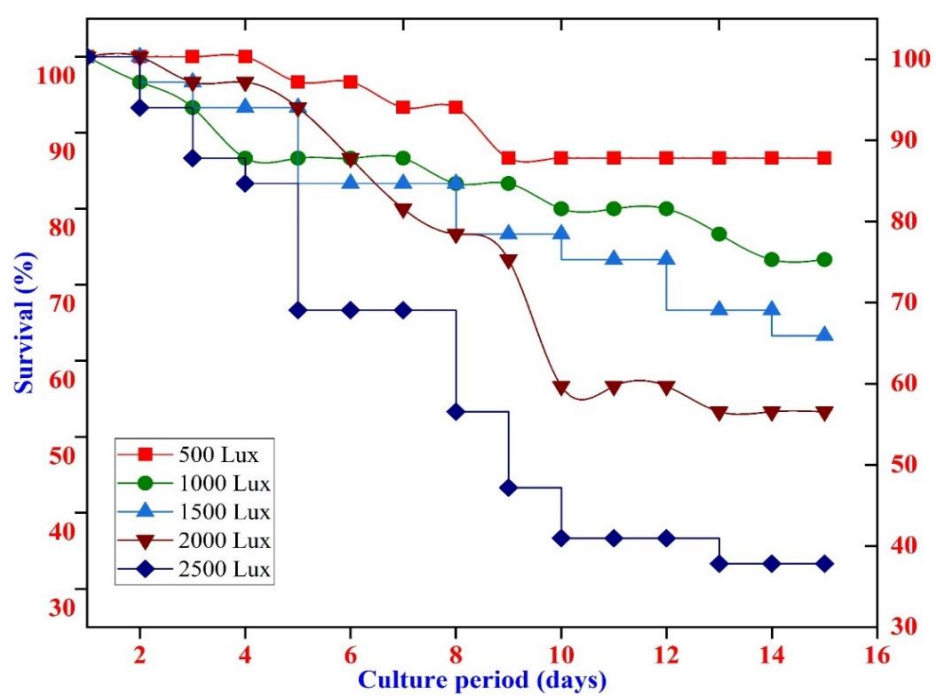


Fig. 31. Survival rate of *O. dissimilis* with reference to light intensity

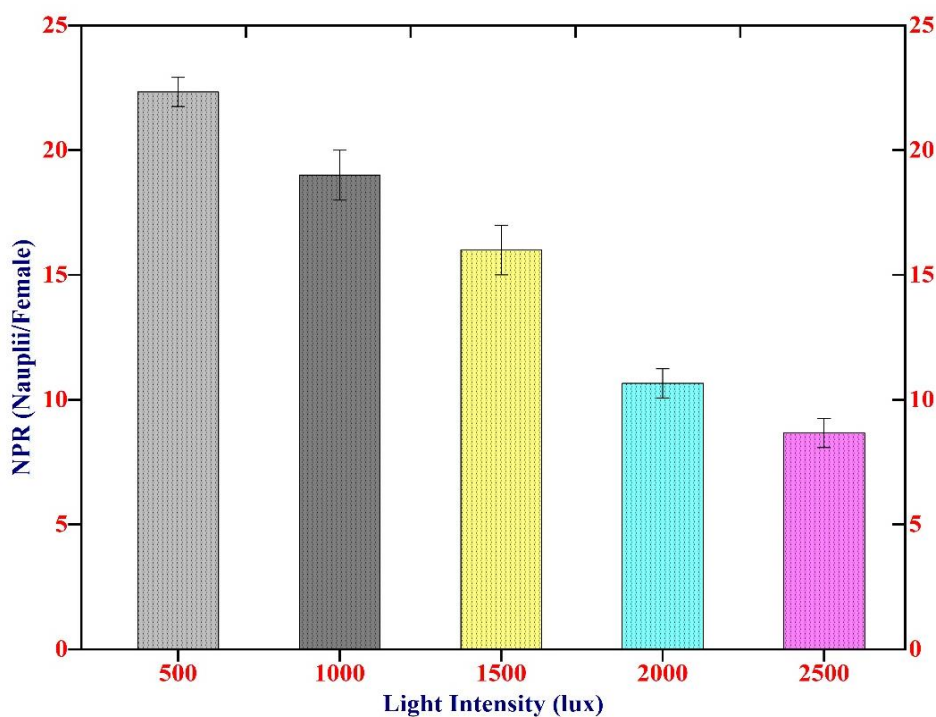


Fig. 32. Nauplii production rate of *O. dissimilis* with reference to light intensity

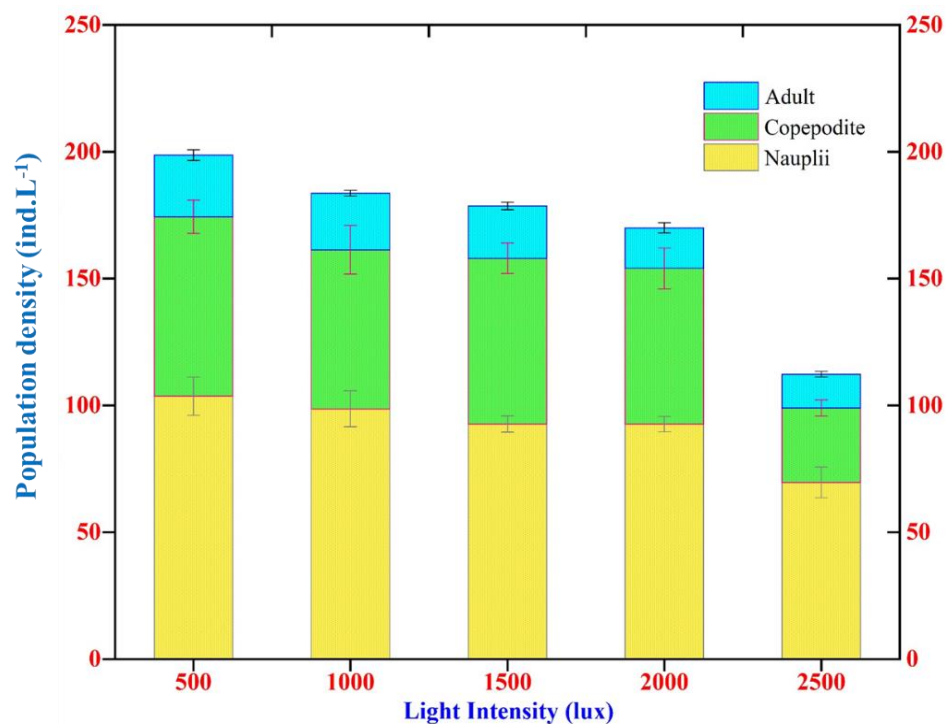


Fig. 33. Population density of *O. dissimilis* with reference to light intensity

Days	Light intensity (lux)				
	500	1000	1500	2000	2500
Development time (N1-N6)	4.2	4.4	4.6	4.8	4.9
Development time (C1-C6) (adult)	9.00	9.4	9.6	9.7	9.9
Total development time	13.2	13.8	14.2	14.5	14.8
Generation time	15.2	15.6	15.8	15.9	16.2

Note: N1-N6: Nauplii 1-Nauplii6; C1-C6: Copepodite 1-Copepodite 6

Table 16: Effect of light intensity on post embryonic development of marine copepod *O. dissimilis*.

Optimization of different feed

Survival rate

In case of different diets experiment, 50% of survival was observed in almost all algal feeds used. However, the maximum survival rate (93.33%) was observed at *I. galbana* followed by *C. marina* (83.33%), mixed algae (76.67%) and *D. salina* (56.67%). The minimum survival rate (60%) was noticed at *T. suecica* (Fig. 33).

Nauplii production rate

The type of feed was found to influence the rate of production of nauplii in almost all the tests performed. The highest production rate (23.66 nauplii/female) was recorded at mixed algal feed which were significantly higher ($P < 0.001$) than *T. suecica* followed by *C. marina* ($P < 0.05$), *D. salina* ($P < 0.01$) and for *I. galbana* ($P > 0.05$) which showed no significant difference. The lowest production rate (16.33 nauplii/female) was noticed at *T. suecica* diet which was significantly lower ($P < 0.001$) than other feeds tested except *D. salina* which showed no considerable difference ($P < 0.05$) (Fig. 34).

Population density

At different algal feed experiment, the maximum peak (361.33 ind. L^{-1}) in population density was obtained in mixed algae which was significantly higher ($P < 0.001$) than the rest of the feeds tested followed by *C. marina* ($P < 0.01$) and *I. galbana* ($P < 0.05$) respectively. The minimum population (267 ind. L^{-1}) was obtained in copepod fed with *D. salina* which was significantly lower ($P < 0.001$) compared to other feeds. There was no significant difference ($P > 0.05$) found between *D. salina* and *T. suecica*. Thus, in all trial, different feed types significantly affect the population density of different life stages of copepod (Fig. 35).

Post Embryonic Development

The shortest duration (4.2 days) for nauplius development (N1-N6) was observed in mixed algae followed by *I. galbana* with 4.3 days. The copepodite to adult development (C1-C6) was shorter in mixed algae with 9 days and longer in *I. galbana* with 9.1 days. The shortest generation time of 14.6 was observed in mixed algae, whereas it was longer in *I. galbana* with 14.7 days (Table 17).

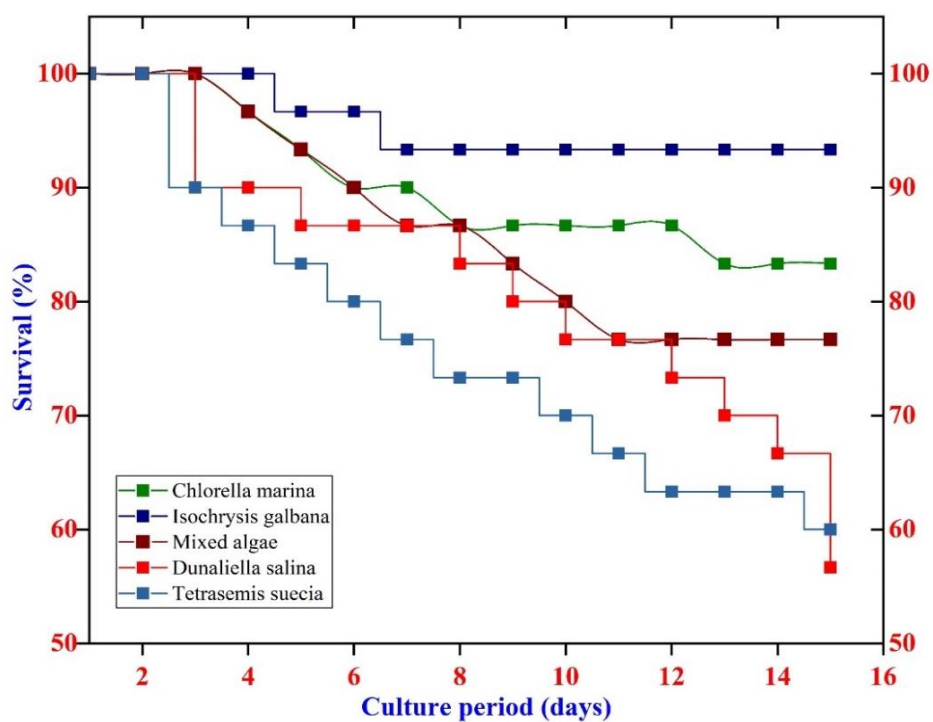


Fig. 34. Survival rate of *O. dissimilis* in respect to diets

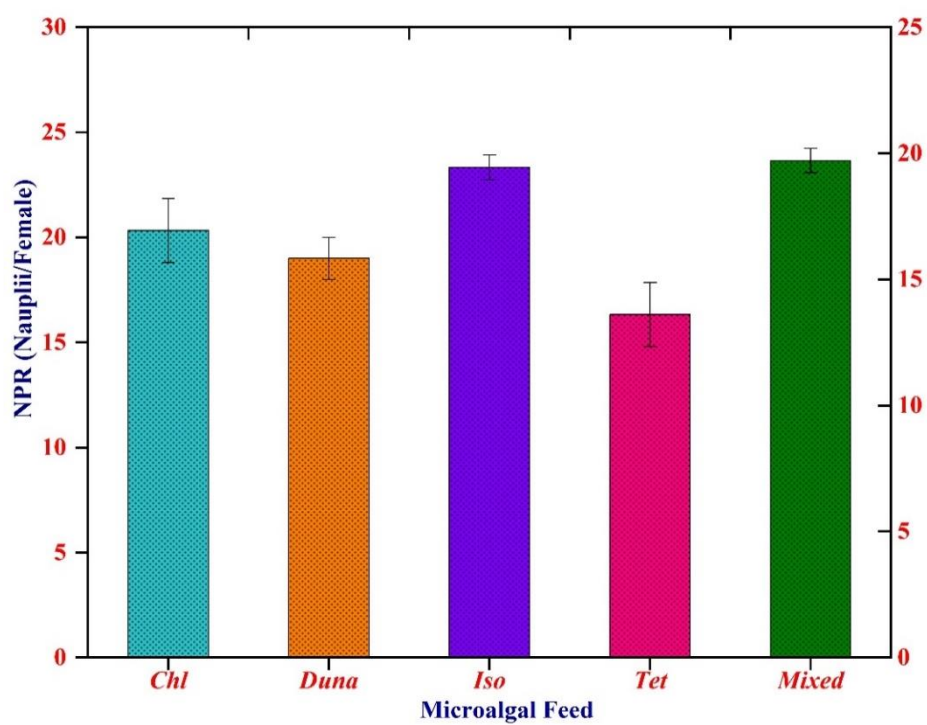


Fig. 35. Nauplii production rate of *O. dissimilis* in respect to diets

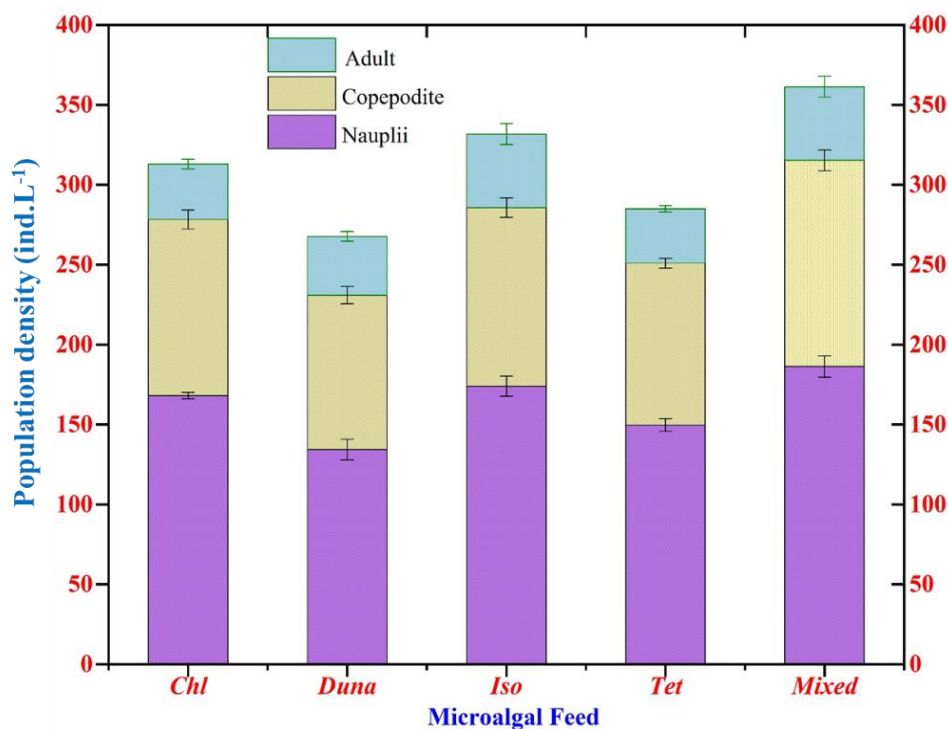


Fig. 36. Population density of *O. dissimilis* in respect to diets

Days	Microalgal feed				
	CHL	DUNA	ISO	TETRA	MIXED
Development time (N1-N6)	4..6	4.8	4.3	5	4.2
Development time (C1-C6) (adult)	9.8	9.4	9.1	9.7	9
Total development time	14.4	14.2	13.4	14.7	13.2
Generation time	15.6	15.4	14.7	14.9	14.6

Note: N1-N6: Nauplii 1-Nauplii6; C1-C6: Copepodite 1-Copepodite 6.

Table 17: Effect of diets on post embryonic development of marine copepod *O. dissimilis*

Optimization of diet concentration

Survival rate

During different concentrations of feed tested presently, there was 50% survival noticed in almost all the concentrations tested except 15,000 cells/ml. There was a gradual decrease in survival percentage starting from the beginning and towards the end of the experiment at low concentration (15000 cells/ml). The maximum survival of 86.67% was

observed at 30000 cells/ml followed by 76.67% at 35000 cells/ml, 73.33% at 25000 cells/ml and 63.33% at 20000 cells/ml (Fig. 36).

Nauplii production rate

The maximum nauplii production rate (21.66 nauplii/female) was noticed at 25000 cells/ml which was significantly higher ($P < 0.001$) than 15000 cells/ml followed by 35000 cells/ml ($P < 0.05$) and there was no significant differences ($P > 0.05$) in concentration found with 30000 and 20000 cells/ml respectively. The lowest nauplii production (15.66 nauplii/female) was observed at low diets concentration (15000 cells/ml) which was significantly lower ($P < 0.001$) than the other concentrations tested (Fig. 37).

Population density

At different feed concentrations, the total highest population density (229.3 ind. L^{-1}) was observed at 25000 cells/ml which was significantly higher ($P < 0.001$) than the rest of the concentrations tested except at 30000 cells/ml which showed considerable significant difference ($P < 0.05$). The lowest population density (162 ind. L^{-1}) was found at 15000 cells/ml which was significantly lower ($P < 0.001$) compared to other concentration tested followed by 35000 cells/ml ($P < 0.01$) and there was no significant difference ($P < 0.05$) in population existed between 20000 and 15000 cells/ml. Thus, in all trial, concentration was significantly affects population density at different life stages (Fig. 38).

Post Embryonic Development

The shortest duration (4.7 days) for nauplius development (N1-N6) was observed in 25000 cells/ml followed by 30000 cells/ml with 4.8 days. The copepodite to adult development (C1-C6) was shorter in 25000 cells/ml with 9.2 days and longer in 15000 cells/ml with 9.9 days. The shortest generation time of 14.8 days was observed in 25000 cells/ml whereas it was longer in 15000 cells/ml with 15.9 days (Table 18).

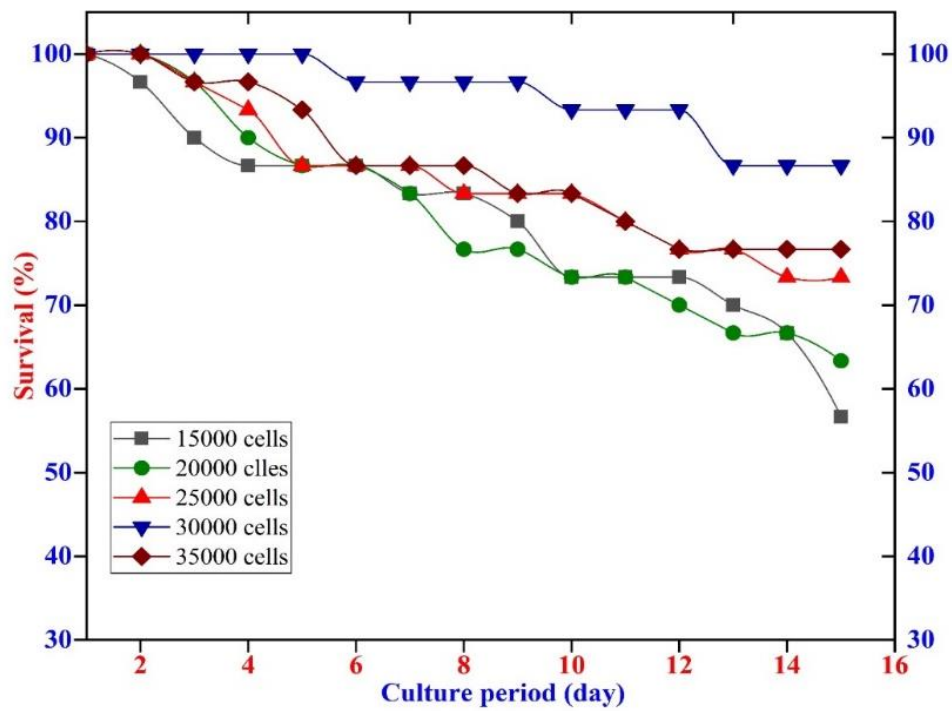


Fig. 37. Survival rate of *O. dissimilis* with reference to diet concentration

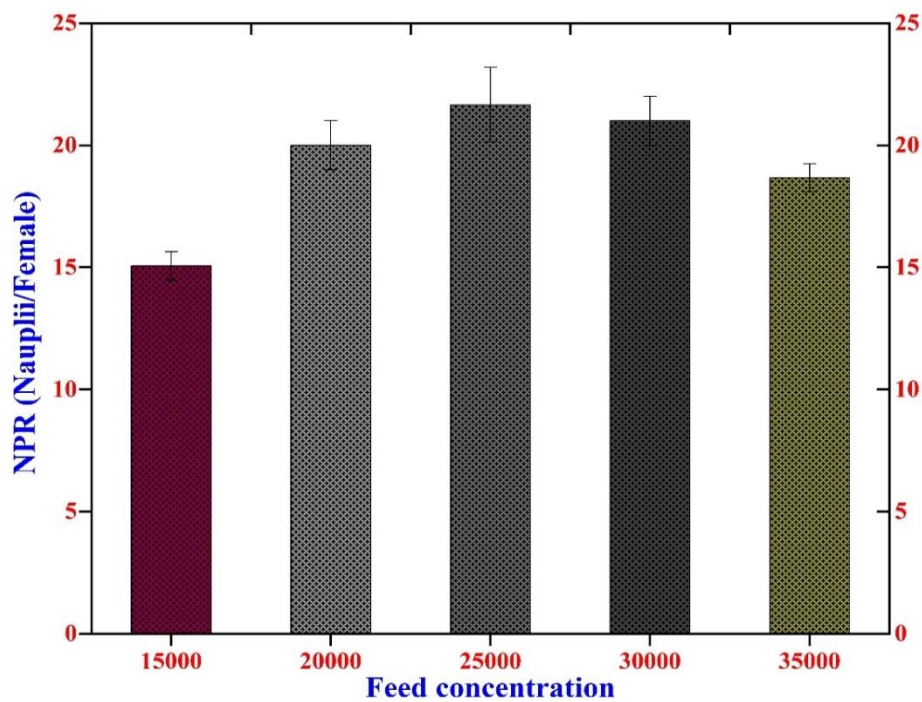


Fig. 38. Nauplii production rate of *O. dissimilis* with reference to diet concentration

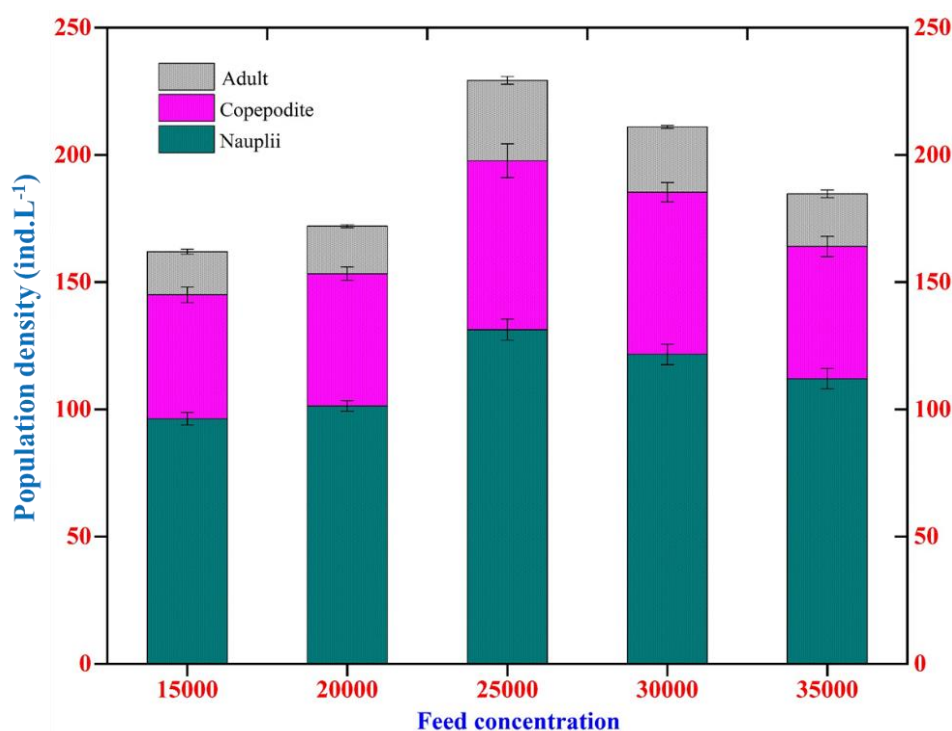


Fig. 39. Population density of *O. dissimilis* with reference to diets concentration

Days	Feed concentration (cells/ml)				
	15000	20000	25000	30000	350000
Development time (N1-N6)	5.4	4.9	4.7	4.8	5.2
Development time (C1-C6) (adult)	9.9	9.7	9.2	9.4	9.5
Total development time	15.3	14.6	13.9	14.2	14.7
Generation time	15.9	15.2	14.8	15	15.7

Note: N1-N6: Nauplii 1-Nauplii6; C1-C6: Copepodite 1-Copepodite 6.

Table 18: Effect of diets concentration on post embryonic development of marine copepod *O. dissimilis*

Discussion

The present results showed that the tested environmental parameters have significant impacts on the survival, nauplii production, population density and post embryonic development of copepod *O. dissimilis*. This experimental study concluded that the cyclopoid copepod *O. dissimilis* was able to tolerate a wide range of salinity region of 25-30 PSU. This species belongs to the family Oithonidae and generally *Oithona* groups are commonly

associated with coastal areas and are abundant in brackishwater habitats. This species was able to survive, produce nauplii and results high population density at the salinity range of 15-35 PSU. There was a maximum mortality, low nauplii production and low population density at low salinity (15 PSU) which might be due to the additional osmoregulation and respiration demands at these salinities (Kimoto *et al.*, 1986; Santhanam 2012).

The present results showed that *O. dissimilis* was able to survive and reproduce better and develop at temperature range from 20 to 36°C as agreed earlier by Walter (1986), Santhanam (2018) Kaviyarsan (2019). The temperature above 30°C results lengthened development time from nauplius to adult stage. Hirche (1997) have also suggested that elevated temperature could impact on reproductive parameters by controlling the metabolic activity of copepods. Apart from other water quality parameters, diet was also played a significant role in reproductive performance of the copepod (Santhanam and Perumal, 2012b; Santhanam *et al.*, 2018). Temperature is often the most important environmental factor affecting the productivity of copepods in natural systems (Rhyne *et al.* 2009; Ananth 2015).

The presently recorded maximum survival, nauplii production rate and total population density can be attributed towards low light intensity of 500 Lux. The increase in light intensity suppressed the production of offspring, survival and growth rate of copepods significantly. The possible mechanism to interpret this result could be due to stress and energy consumption by copepods for their living under intense light conditions. In this present study, it was clearly found that increased light intensity negatively impacted the production and development rate of copepod *O. dissimilis*. Omori and Ikeda (1984) proved that changing illumination affects the endocrine activity in marine copepods and also directly affects reproduction, maturation and impacts in release of eggs, hatching, and death in copepods. (Kaviyarasan *et al.*, 2019; Farhadian, *et al.*, 2014).

Food quality affects the development and fecundity in the copepods. Algal diets have significantly influence the survival rate, nauplii production and population of *O. dissimilis*. The survival, nauplii production and total population density of copepod in my experiment confirmed that highest rate was achieved by copepods fed with mixed algae. This might be due to the fact that applying monodiets may cause nutritional deficiencies of one or more essential nutrients. To reduce this risk, several researches have recommended the use of mixed diets as the combined nutrient contents would satisfy the nutritional requirements of the target species (Brown et al., 1989; Smith *et al.*, 1992; Santhanam and Perumal 2012).

In response to feed concentration, the copepods had the maximum survival rate, nauplii production and higher population density supplied with higher concentration of algal cells but the ratio has been declined in copepods supplied with low concentration of algal cells (15000 cells/ml) might be due to food scarcity. Since, food is one of the important factors to enhance better growth and density of copepods in the culture systems, the copepods population increased in direct proportion to the increased food supply and poor results were obtained at low food concentration (Schipp *et al.*, 2006; Santhanam and Perumal 2012).

As conclusion, the environmental parameters are highly important for the wellbeing of live feed production. In this study, it was established that *O. dissimilis* cultured under different levels of salinity, temperature, pH, light, feed and feed concentration can survive and reproduce very well. The salinity of 25 PSU, 15-35°C of temperature, 8 pH and 500 lux of light and mixed diet with moderate or high diets concentration provide better survival, population and high nauplii hatching for this candidate species which could be a suitable live feed for aquaculture. The information gathered in our experiment can be used to develop improved, commercial scale culturing system for copepod *Oithona dissimilis* in future.

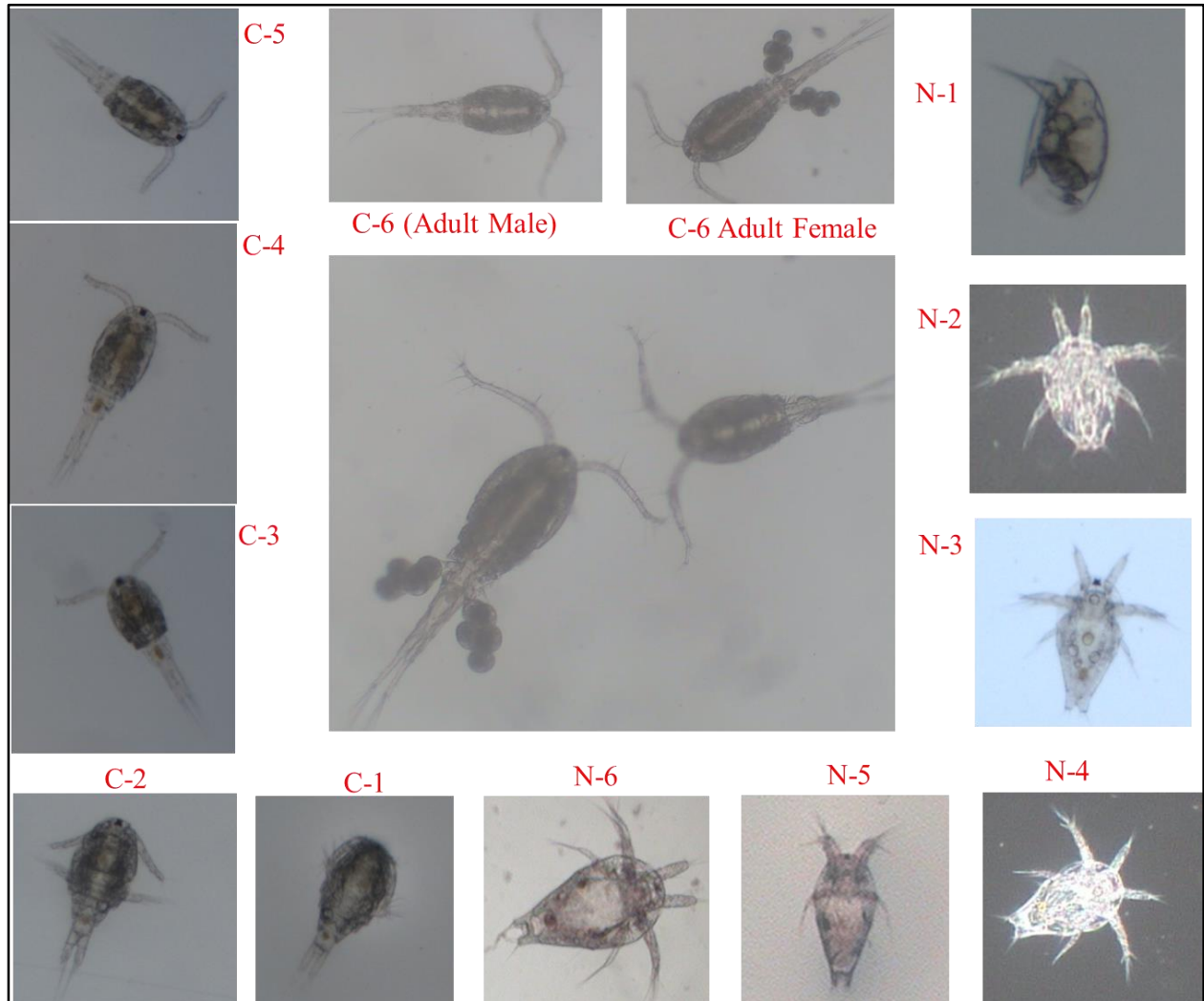


Plate 13. Post-embryonic developmental stages of copepod *O. dissimilis*

Chapter V

8. Impact of Microplastics Ingestion on Physiology of Marine Copepod *Oithona dissimilis*

Introduction

In recent years, following increased awareness of microscopic debris occurring within the oceanic atmosphere, studies have been carried out to assess the potential for microplastic particles to enter and pass through the food chain. Subsequently, the effects of such ingestion upon the individual had been assessed plastic pollution is one of the most challenging and having a serious societal concern in the world today. Being an essential commodity, with multipurpose application in our day-to-day life, plastic has been considered as an indispensable part of human life. Some key qualities like flexibility, durability, easy portability and malleability lead to its widespread usage in agriculture, fisheries, personal care products, textiles, and all other sectors or industries. The global production of plastics has been increased rapidly and already reached 359 million tonnes in 2018 (Europe, 2019). Improper management of these plastics has a devastating effect on the environment and its accompanying living creatures. The increasing and continuous usage of plastics are directly proportional to increase the pin plastic waste. The plastic trashes entering the marine environment almost account for 60 to 80 % of marine garbages (Pitt *et al.*, 2018).

Plastics are made up of high molecular polymers that are used widely in all walks of life. The foremost commonly used plastics are High-Density polyethylene (HDPE), Low-Density Polyethylene (LDPE), polypropylene (PP), polymeric amide (PA), polyvinyl chloride (PVC), and polystyrene (PS) (Nava and Leoni, 2021). Due to various

environmental factors, these plastics undergo fragmentation and resulted in the formation of macro, meso, and microplastics (Zhang *et al.*, 2021). Among this, Microplastics (MPs) prevalence within the environment has been documented in virtually within almost every crevices on the earth, from open to confined ocean environment, from surface water to sea sediments, from the equator to the polar regions, from drinking water to wastewater and even present in the human placenta (Ragusa *et al.*, 2021; Peng *et al.*, 2017). Polystyrene (PS), a thermoplastic polymer characterized by highly transparent, durable and can be easily dyed and used for the production of CDs, toys, toothbrushes and styrofoam. Styrofoam is widely used in manufacturing of some food containers like trays, plates and cups and for packaging materials (Kik *et al.*, 2020). PS is considered as a model microplastics in majority of the studies focussing on the effect of characteristic particle surfaces on various biological parameters, due to their easy synthesizing nature over a broad range of sizes (Loss *et al.*, 2014). However, the knowledge on the toxicity and effect of polystyrene MPs on the aquatic marine invertebrate is inconsiderable and very scarce in India.

The abundance of plankton is directly associated with higher trophic levels which influences the entire ecosystem and it also interacts with other organisms. Among different groups of plankton copepods embody as an important alternative live-feed in marine fish population which improves survival, growth and development of fish larvae (Hansen, 2017). They are used as bio- indicators of environmental conditions, which reflect on the ecosystem status and also in the living component, abundance of predators. They also occupy a crucial position in the food chain. Fragmentation and prolonged degradation of large plastic wreckages results in MP in the form of plastic granules, beads, fragments, and fibres. The abundance of micro-plastics influenced by tide, wind and wave action the effects of upwelling and oceans currents also affect the same. Nowadays the highest waterborne diseases were reported due to high concentrations of micro-plastics. The bioavailability of

marine organisms was affected by micro-plastics including small planktonic organism to higher organism fish. Even they also affect the benthic organisms (polychaetes). Consumption of microplastics can results in adverse health impacts which includes reduced feeding, loss of energetic reserves, hepatic stress, reduced fecundity, oviposition and survival rate. The toxic elements of MPs can transfer and adhered in different substrates which possess waterborne pollutants to organisms. They may also have wider ecological impacts even in colonization of microbial population. The components of MPs can able to amend marine nutrient cycle by altering the properties of copepods which have a key role in marine ecosystem. Hence, the present attempt made on the effect of microplastics ingestion on survival, nauplii production, and population density of copepod *Oithona dissimilis*.

Materials and methods

Microplastic ingestion experiment in *O. dissimilis*

Polystyrene microplastics of three different sizes, such as Latex beads carboxylate-modified polystyrene fluorescent yellow-green (aqueous suspension, 2.0 µm mean particle size), Latex beads, sulphate-modified polystyrene fluorescent orange (aqueous suspension, mean particle size 0.5) and amine modified fluorescent orange beads (aqueous suspension, 0.1 µm mean particle size) (Sigma-Aldrich, USA) were purchased from a private shop.

Marine copepod *O. dissimilis* used for the MPs ingestion experiment was taken from Marine Planktonolgy and Aquaculture Laboarty, Department of Marine Science, Bharathidasan University. Difference sizes such as, 2, 0.5 and 0.1 µm green, orange florescent microplastic beads were used for the experimental study. Copepod *O. dissimilis* fed with only *I. galbana* served as control. Copepods were ingested with lµl of MPs and their survival rate, nauplii production rate, population density and post embrynoci development was determined in control and experimental copepod.

Effect of MPs ingestion on survival rate of *O. dissimilis*

The survival rate of *O. dissimilis* was analyzed for the period of 15 days. A 5 numbers of healthy *O. dissimilis* were picked up from the stock culture and stocked in 100-ML beaker filled with sterile seawater. There are seven different MPS ingestion experiment were made viz; 1) 2.0 μm MPs; 2) 0.5 μm MPs; 3) 0.1 μm MPs; 4) 2.0 μm MPs+ISO; 5) 0.5 μm MPs+ ISO; 6) 0.1 μm MPs+ ISO and 7) ISO (control). Latex beads, amine-modified polystyrene fluorescence yellow green and orange MPs were used for MPs ingestion experiment. The copepod specimens remaining in control and test was recorded daily. Dead copepods are removed from treatments. The survival rate of copepod in respect to experimental and control groups were measured by subtracting the final density of copepods from initial density of copepods.

Effect of MPs ingestion on Nauplii Production Rate (NPR) of *O. dissimilis*

For determining the effect of MPs ingestion on NPR, five individual *O. dissimilis* female with viable egg sacs were stocked in a glass test tube containing 20 ml of filtered seawater. The seven different MPs ingestion experiment including control were done. The copepod was examined at regular intervals (every 2 hour) 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.

Effect of MPs ingestion on population density of *O. dissimilis*

A 5 pair of adult copepods (1:1 Male: Female) were stocked in to each 100-ml beaker filled with sterilized seawater. The copepods were fed once in every two days with MPs and ISO (algae). No copepods were removed from the containers during the experimental period of 15 days. On the final day, all the copepods including nauplii, copepodite and adult were filtered through 48 μm sieve. The total copepods produced over the period of experiment

were counted using Sedgewick rafter counter under the microscope. The number of nauplii, copepodite and adult copepods produced in control and test group were counted separately.

Effect of MPs ingestion on post embryonic development *O. dissimilis*

To assess the developmental period, a gravid female of *O. dissimilis* was stocked in a clean test tube filled with filtered seawater. The test and control groups copepods were ingested with MPs and algae respectively. The copepod was checked for hatching at regular intervals. After hatching of the nauplii, the female was removed from the plate and the nauplii were observed for further development. In this experiment, the time taken for eggs to nauplii hatch (embryonic development), the development of nauplii stages (NI-NVI) in to copepodite stages (CI-CVI) were noted. The generation time (egg to egg) was also recorded. Few nauplii from each treatment were cultured separately until they spawn to determine the generation time.

Results

Treatments

For the ingestion experiments, *I. galbana* and microplastic stock concentration such as 30,000 cells/ ml and 18.27×10^7 per ml (microplastic-enriched solutions) respectively was used. During the study period, the different sized microplastics mixed with algal diet and were given to copepod.

Survival rate

In the present study, the average survival of *O. dissimilis* was 96.66% with no significant difference between control and microplastic treatment ($P < 0.005$) on the 6th day. The survival rate of 0.1 μm microplastic exposed copepods dropped to 63.33% followed by 2.0 and 0.5 μm respectively. From day 6 onwards, the survival rate of microplastic exposed copepods declined significantly ($P < 0.005$). By the final (Day10-15) survival rate of control

was decreased to 93.33% while 2.0 μm microplastic exposed copepod dropped to 46.66% survival. On the final day of experiment the survival rate of 0.5 μm microplastic exposed copepod was significantly showed 56.66% survival closely matching the survival rate of 0.1 μm microplastic exposed copepods ($P < 0.05$). (Fig. 39)

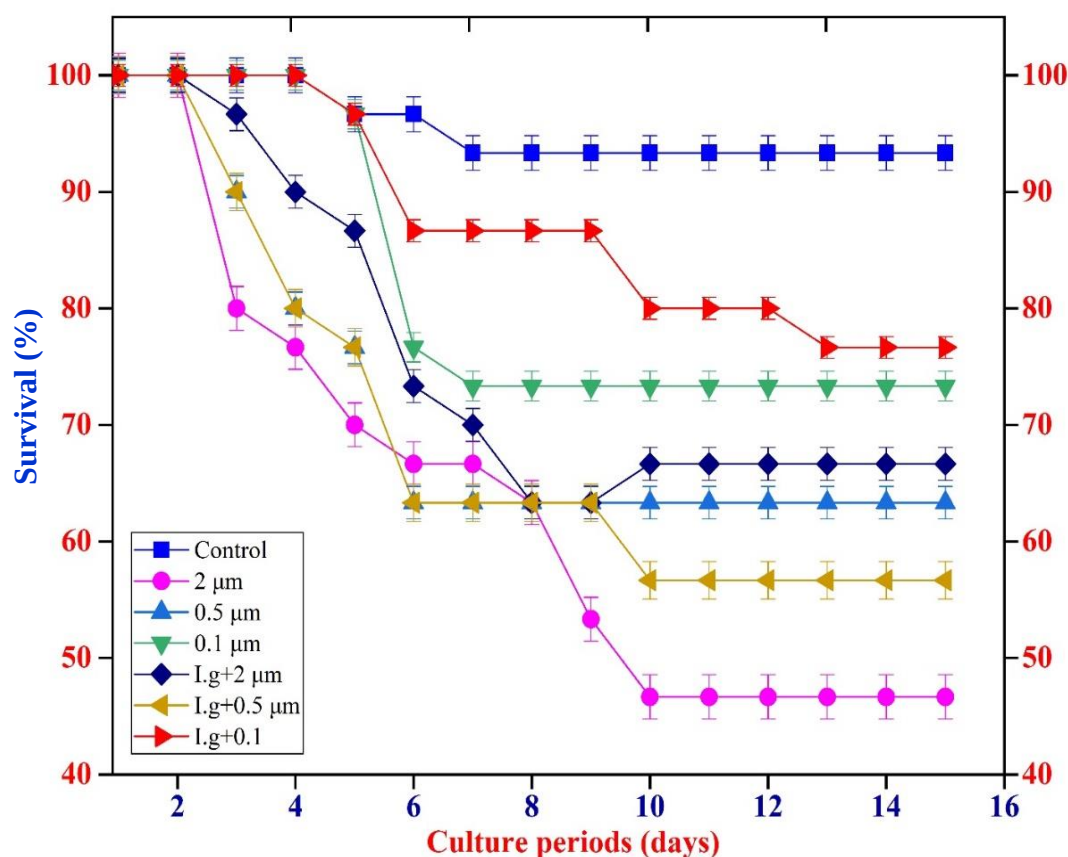


Fig. 40. Effect of MPs ingestion on survival rate of *O. dissimilis*

Nauplii Production Rate

In the present study the rate of nauplii production was high in control copepods whereas the production rate was low in microplastic ingested copepods. The high rate of nauplii production was noticed in control *O. dissimilis* 21.89 ± 0.89 whereas the low rate of nauplii production was found in treatment groups (12.56 ± 0.89). There was a significant

($P < 0.005$) differences in nauplii production rate between control and microplastic-exposed copepod (Fig. 40).

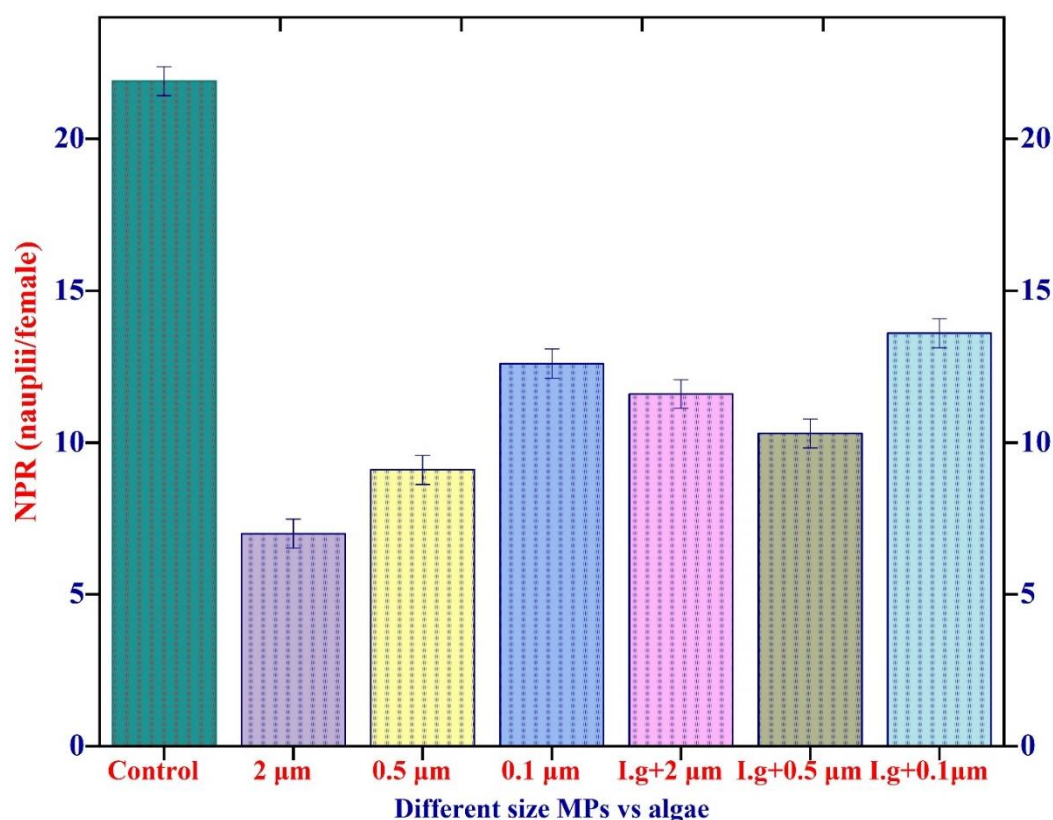


Fig. 41. Effect of MPs ingestion on nauplii production rate of copepod *O. dissimilis*

Population Density

Nauplii

The microplastic exposed *O. dissimilis* produced significantly lower density of nauplii ($P < 0.005$) compared to control (Fig. 41). After 15th day exposure period, 2.0 µm microplastics exposed *O. dissimilis* results least nauplii production of 38 ± 22 nauplii compared to control which yield 177.6 ± 2.75 whereas 0.5 µm and 0.1 µm microplastic exposed copepods showed 56.78 ± 1.73 and 72.44 ± 2.73 nauplii respectively.

Copepodite

As compared to control, 2.0 μm microplastic exposed *O. dissimilis* produced less number of copepodite (38.22 ± 1.94) followed by 0.5 μm and 0.1 μm microplastic exposed *O. dissimilis*. However, the duration from nauplii phase to copepodite formation was substantially longer with 2.0 μm ingested copepod compared with control. The smallest size beads (0.1 μm) had no effect on generation time, whereas 0.5 μm MPs caused substantial change in *O. dissimilis* generation time (Fig. 41).

Adult

Across treatments, the control group had the highest number of adult copepods (41.56 ± 0.85), whereas the 2.0 μm microplastic exposed *O. dissimilis* had the lowest number (24.44 ± 1.92) followed by 0.1 μm (40.89 ± 2.48) and 0.5 μm (34.56 ± 1.73) microplastic (Fig. 41)

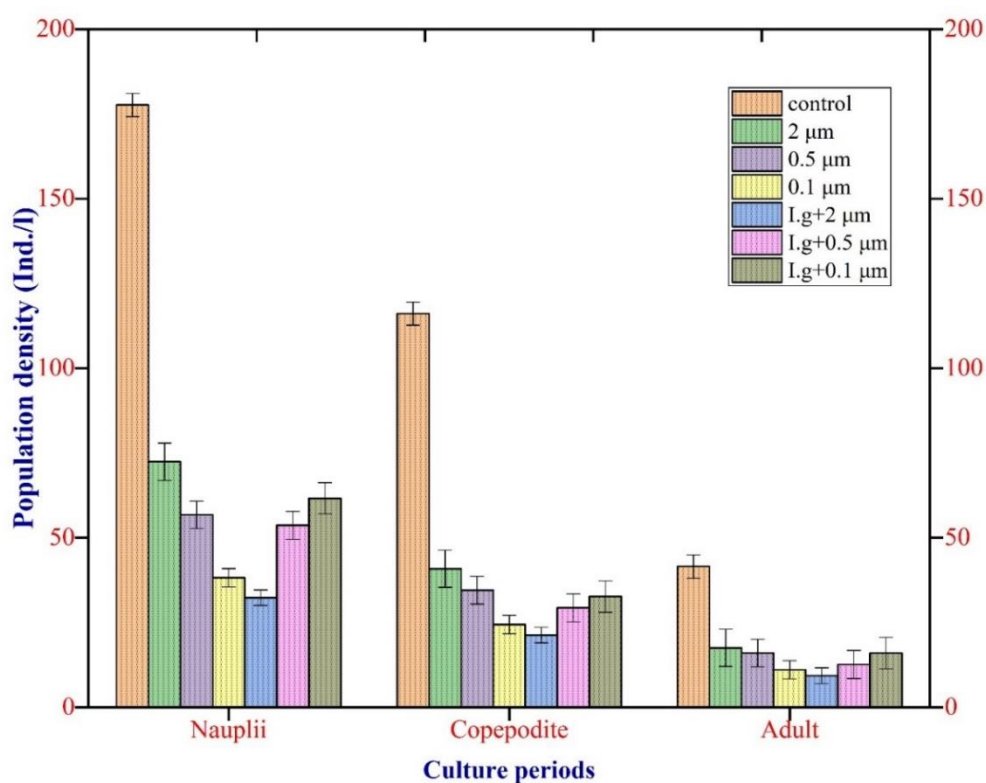


Fig: 42. Effect of MPs ingestion on population density of *O. dissimilis*

Effect of MPs ingestion post embryonic development of *O. dissimilis*

The shortest duration for nauplius development (N1-N6) was observed in control copepod with 4.3 days followed by 0.1 µm MPs exposed copepod with 5.2 days. The longest duration (6.6 days) for nauplius development (N1-N6) was observed in 2.0 µm MPs exposed copepod followed by 0.5 µm MPs copepod exposed with 5.9 days. The shortest duration for copepodite development (C1-C6) was observed in control (*I. galbana*) with 8.9 days followed by 0.1 µm MPs exposed copepod (9.7 days). The longest duration for copepodite development (C1-C6) was observed in 2.0 µm MPs exposed copepod (10.4 days) followed by 0.5 µm MPs exposed copepod (10.2 days). The shortest generation time of 13.5 days was observed in control copepod followed by 2.0 µm MPs exposed copepod (14.7 days), 0.1 µm MPs exposed copepod (14.9 days) and 0.5 µm MPs exposed copepod (16.1 days) (Table 19).

Days	Control vs MPs						
	Control	0.1 µm	0.5 µm	2.0 µm	1.g+1 µm	1.g+0.5 µm	1.g+2 µm
Development time (N1-N6)	4.6	5.2	5.9	6.6	5.8	5.6	6.2
Development time (C1-C6)	8.9	9.3	10.2	10.4	9.5	9.8	10
Generation time (GT)	13.5	14.9	16.1	17.0	14.6	15.8	16.6

Note: N1-N6: Nauplii 1-Nauplii6; C1-C6: Copepodite 1-Copepodite 6.

Table 19: Effect of MPs ingestion on post embryonic development of marine copepod *O. dissimilis*

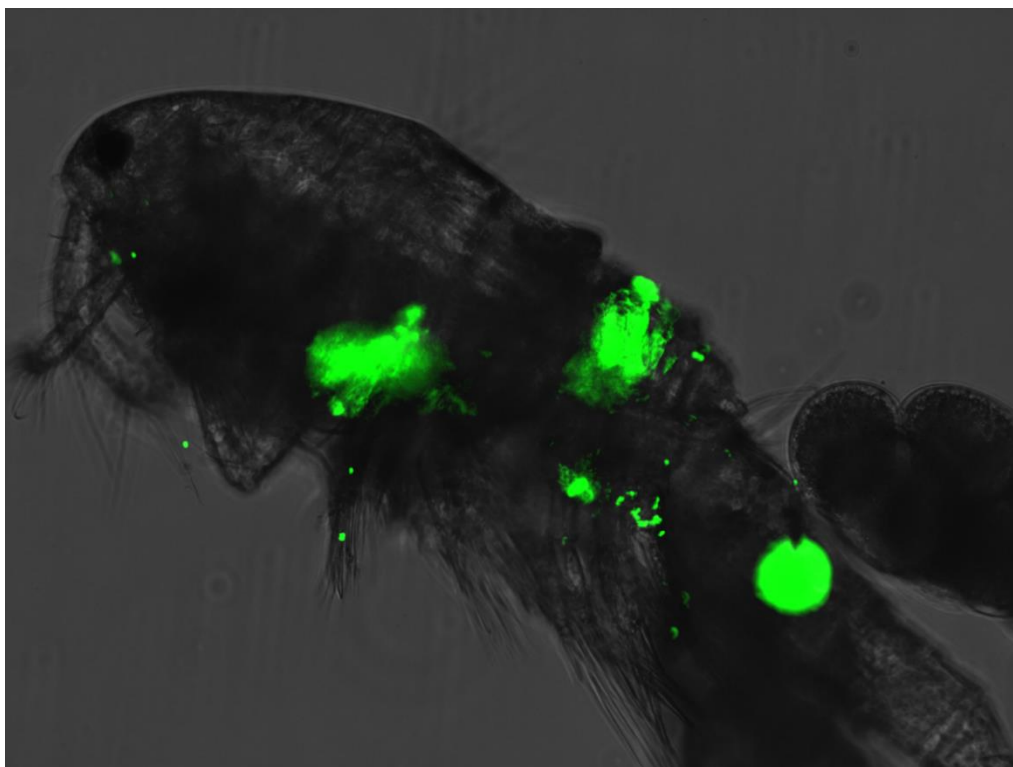


Plate 14. The accumulation of MPs (2.0 μm) in the gut of *O. dissimilis*

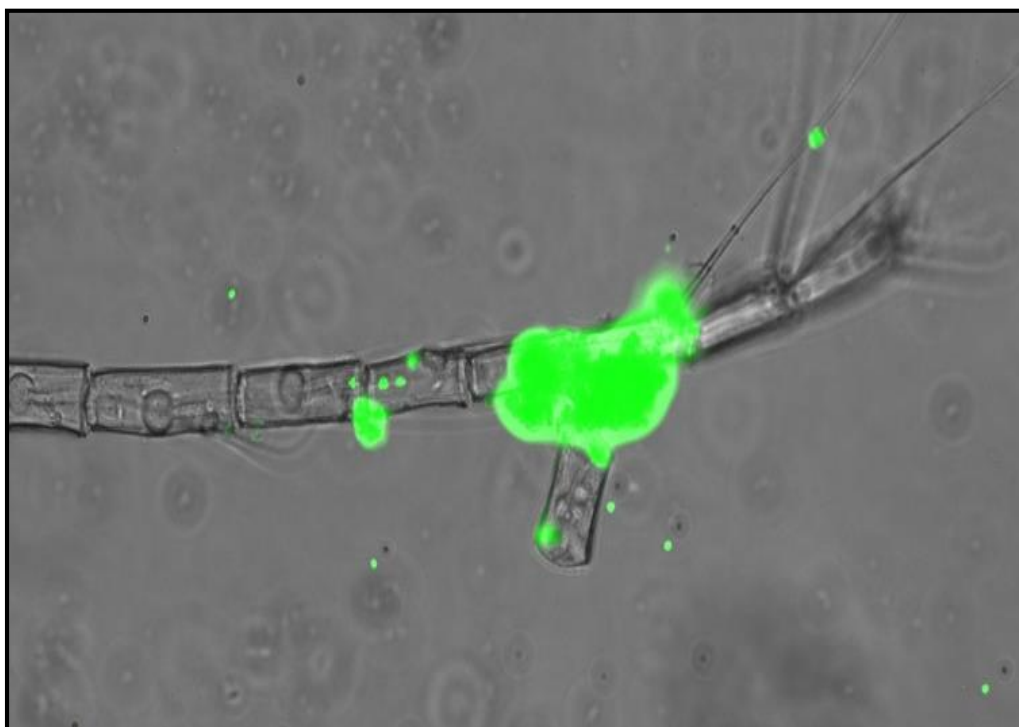


Plate 15. The accumulation of MPs (2.0 μm) in the antenna of *O. dissimilis*

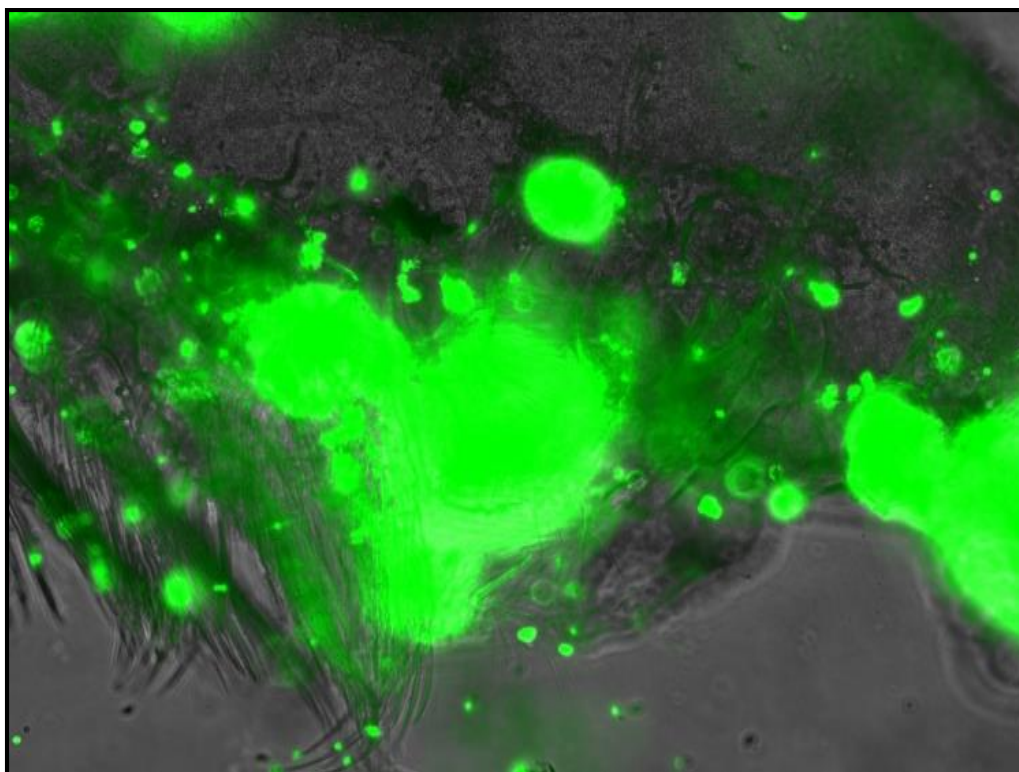


Plate 16. The acumulation of MPs (2.0 μm) in the leg region of *O. dissimilis*

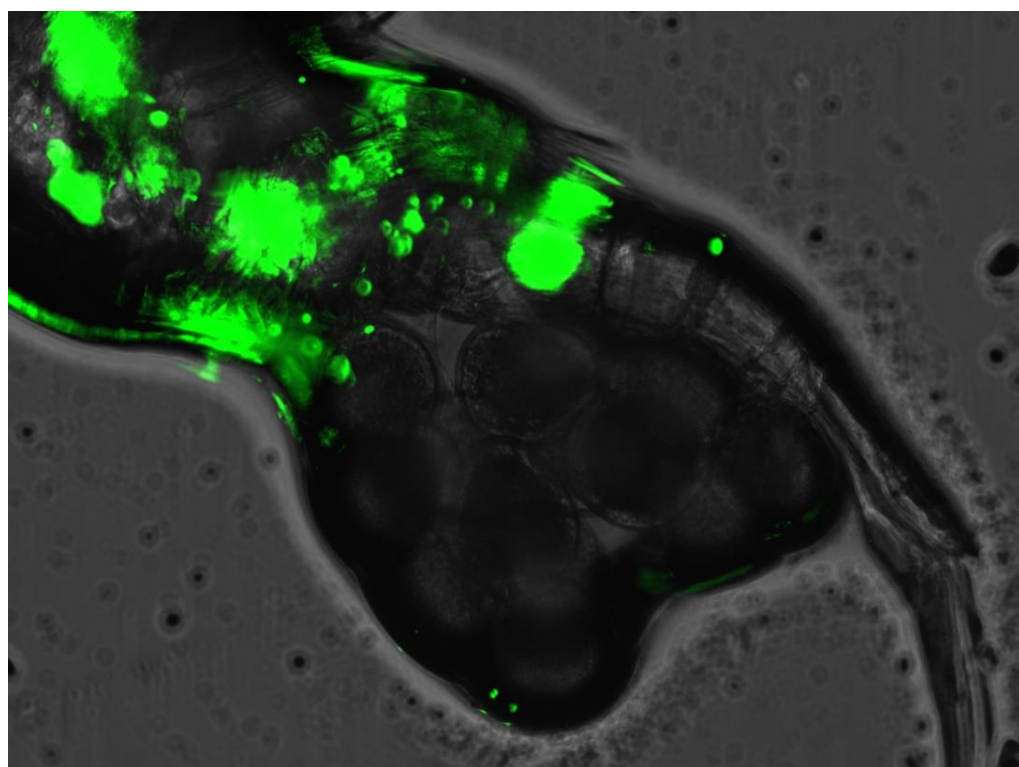


Plate 17. The accumulation of MPs (2.0 μm) in the urosome of *O. dissimilis*

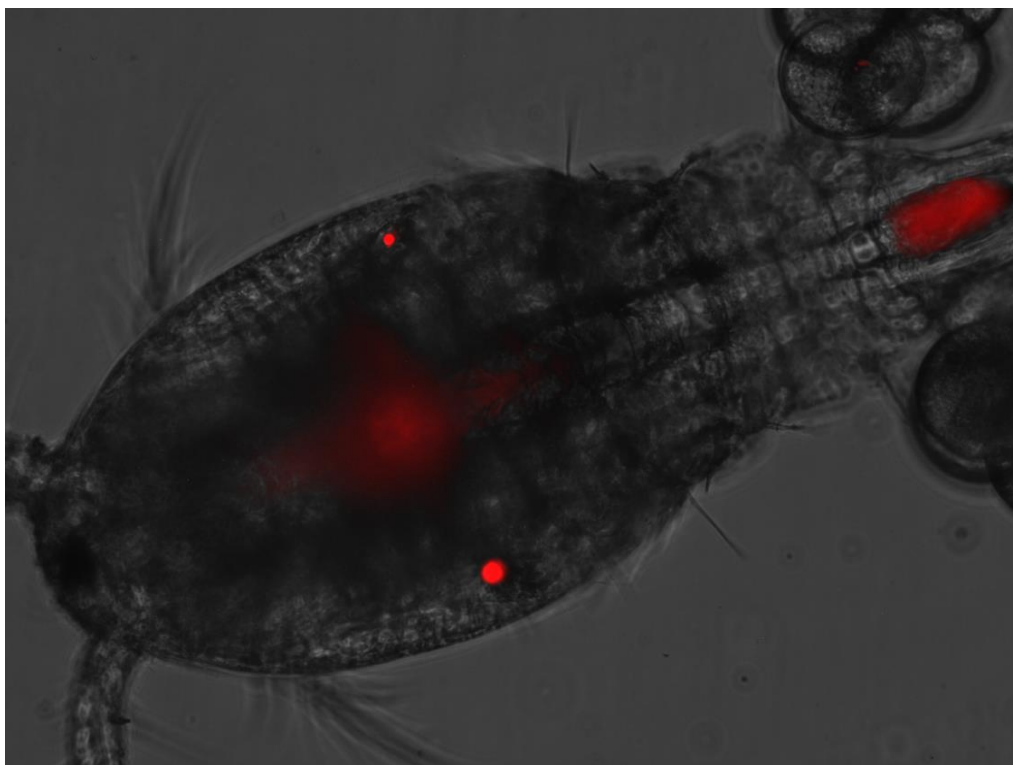


Plate 18. The accumulation of MPs (0.5 μm) in the gut of *O. dissimilis*

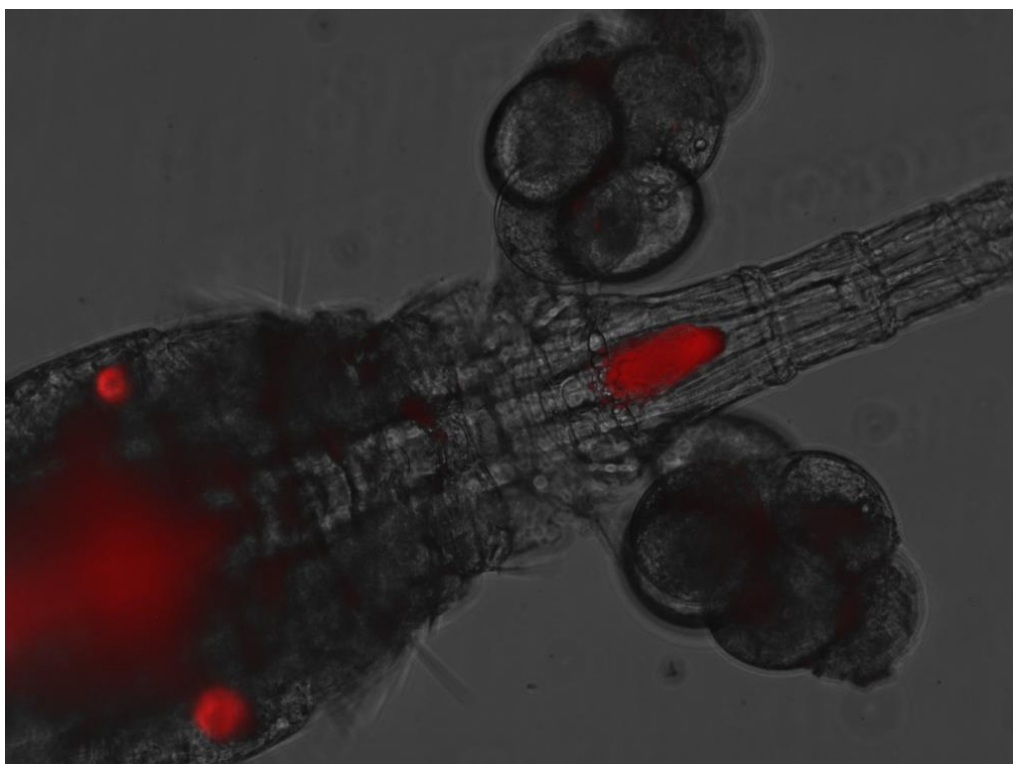


Plate 19. The acumulation of MPs (0.5 μm) in the gut and urosome of *O. dissimilis*

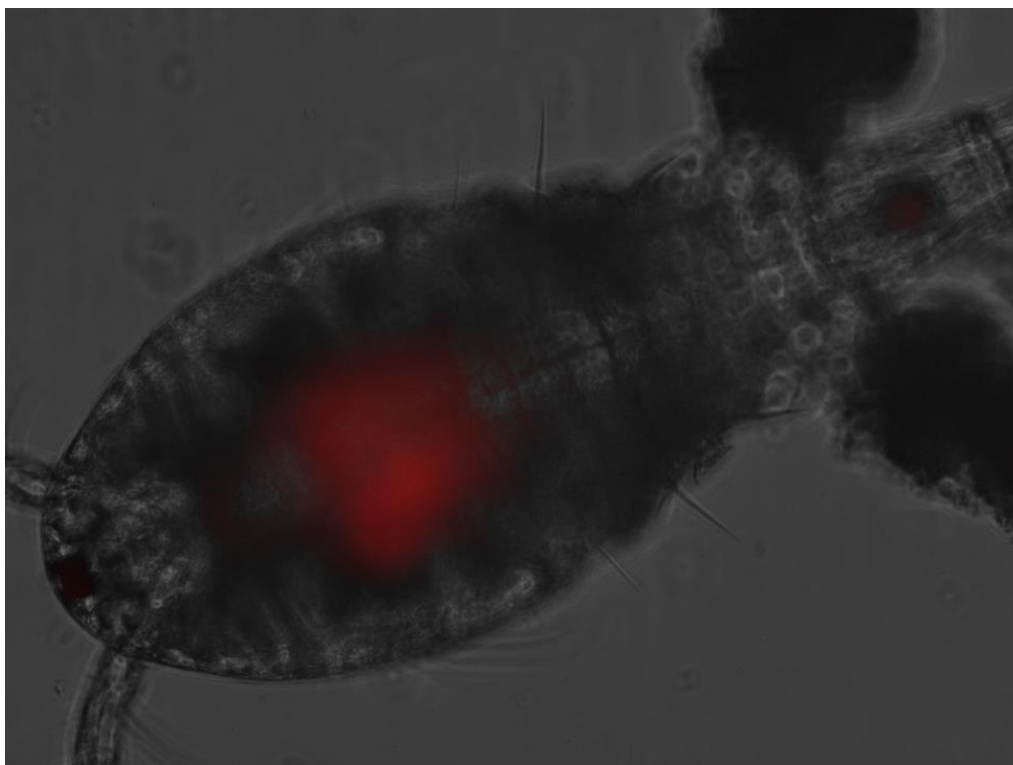


Plate 20. The accumulation of MPs (0.1 μm) in the gut of *O. dissimilis*

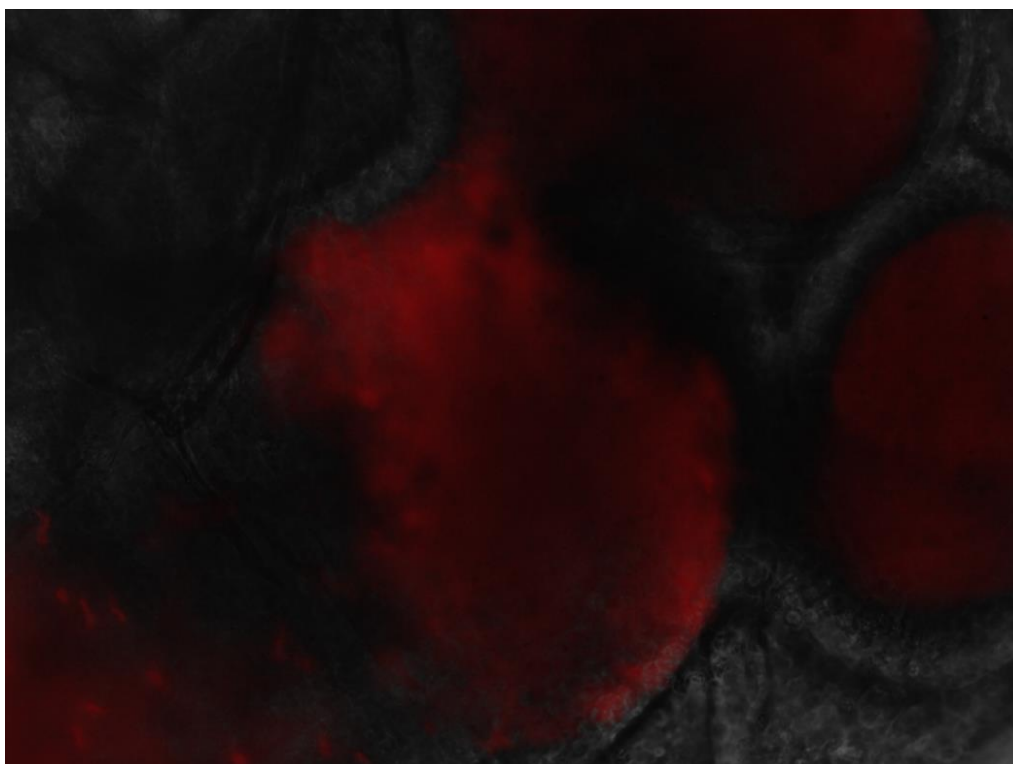


Plate 21. The accumulation of MPs (0.1 μm) in the egg sac of *O. dissimilis*

Discussion

Microplastics (MPs) have been found in many parts of the marine food web, especially in the water column with several impacts on the marine copepod (Cole *et al.*, 2015). This study investigated the effects of ingestion of 0.1, 0.5, and 2 μm microplastics that might have adverse effect on the filtration ability of copepod *O. dissimilis*. The present results showed that the copepod can ingest microplastics (0.1, 0.5, and 2.0 μm diameter) in the absence of natural food. Microplastics were unknowingly ingested via filter-feeding and later egested in faecal pellets, typically within a matter of hours. Microplastics were found to be trapped between the external appendages of copepods owing to their accumulation on the external surface.

The microscopic images of copepod clearly showed that 0.1, 0.5, and 2.0 μm polystyrene MPs clustered within the alimentary canal and aggregated between the setae and joints of external appendages. The ingestion of 0.1, 0.5, and 2.0 μm polystyrene beads reduced the nauplii production, and survival rates in copepod *O. dissimilis*. The initial effects upon the organism are likely to occur in the digestive tract, or gut, of the animal. In ingestion studies examining the uptake of polystyrene spheres by copepods, particles had the potential to be retained in the gut for up to 7 days (Cole *et al.*, 2013). Adherence to feeding appendages (highlighted in image, Plate 21-28) and swimming legs, appeared common across all test plastics, and copepods as well as, cases of adherence to the antennae, carapace, and urosome. Such adherence, as described by Cole *et al.* (2013) who reported the potential adverse impact of MPs ingestion on feeding, predator avoidance, and mating of zooplankton.

Microplastics adherence to body parts might alter the individual's buoyancy and limited swimming ability may increase the chances of predation due to altered movement causing an increased disturbance in the water, so enabling predators to detect the copepod

more easily, or reducing the effectiveness of avoidance strategies such as the escape jump displayed by a range of nauplii and zooplankton prey (Jakobsen, 2001; Titelman & Kiørboe, 2003). The microplastics were egested in a number of hours, at a rate similar to that of natural prey (Cole *et al.*, 2013). Thus, egestion may provide a potential source of secondary uptake via coprophagy of faecal pellets by other zooplankton or marine species. The retention of microplastics has the potential to cause physical harm to the individual. Such gut retention and blockages may negatively affect the manner in which copepod species ingest and subsequently digest food, and potentially may increase the likelihood of chemical effects being endured by the individual.

Similarly, Cole *et al.* (2013) reported that *Temora longicornis* and *Centropages typicus* ingested microplastics with different sizes such as 7.3, 20.6, and 30.6 μm beads and *Acartia clausi*, *Calanus helgolandicus* ingested 7.3 μm beads affected the feeding and survival rates of the animals. The present study demonstrated that the minimum survival and nauplii production rate was found in the microplastic ingested copepod. Prolonged exposure to the microplastics resulted in less nauplii production with reduced hatching success and the survival rate was also reduced within 4-5 days in *O. dissimilis*. The present findings showed that microplastics can impede copepod feeding and reduce the nauplii production rate and survival. These effects were most noticeable at 3-4 days after the ingestion of microplastics into the organisms. Lee *et al.* (2013) found that when copepod exposed to 0.5 and 6 μm microplastics, the number of nauplii hatched from eggs produced by the benthic copepod *Tigriopus japonicus* was reduced. Similar results have been observed by Bonnet *et al.* (2005) and Cole *et al.* (2013, 2015).

Kunckey *et al.* (2005) also observed that *I. galbana* fed *Acartia sinjiensis* showed better development. The lowest Development time (DT) was obtained with the diet *Nitzschia closterium* for *T. biminiensis* by Pinto *et al.* (2001). The result showed that DT (N1-N6 4.6

days, C1-C6 8.9 days) was shortest with the diet *I. galbana*. DT was a very slow in 2 µm ingested copepod the microplastics (N1-N6 8.9 days, C1-C6 10.4 days). The Generation time (GT) of *Oithona dissimilis* was comparatively shorter (13.5 days) in control group than the MPs ingested copepod. Very slow GT was observed in 2 µm MPs ingested copepod (17 days) followed by 0.5 µm MPs ingested (16.1 days) and 0.1 µm MPs ingested copepod (14.9 days) might be due to lack of essential nutrition as feeding is negatively affected (Payne and Rippling, 2000; Kunckey *et al.* 2005; puello-cruz *et al.*, 2009).

Chapter VI

9. Impact of Microplastics Ingestion on Nutritional Profile of Marine Copepod *Oithona dissimilis*

Introduction

Plastic production has increased exponentially since the early 1950s and reached 322 million tons in 2015 excluding synthetic fibers which accounted for an additional 61 million tons in 2015. It is expected that production of plastics will continue to increase in the future and production levels are likely to double by 2025. Inadequate management of plastic waste has led to increased contamination of freshwater, estuarine and marine environments. It has been estimated that in 2010 between 4.8 million to 12.7 million tons of plastic waste entered the oceans. Abandoned, lost or otherwise discarded fishing gears are considered as the main source of plastic waste by the fisheries and aquaculture sectors, but their relative contribution is not well known at regional and global levels. There is increasing scientific and societal concern about the effects of microplastics (MPs), commonly defined as plastic particles with sizes below 5 mm (Betts, 2008; Fendall and Sewell, 2009; Hidalgo-Ruz *et al.*, 2012), on freshwater and marine organisms (Kubota, 1994; Gregory and Ryan, 1997; Yoon *et al.*, 2010; Zarfl and Matthies, 2010; Kako *et al.*, 2011, 2014; Maximenko *et al.*, 2012; Isobe *et al.*, 2014).

Plastic is the major form of marine debris in our ocean. Plastic debris consists of varying shapes and sizes, but those that are less than five millimeters in length or about the size of a sesame seed are called “Microplastics”. Microbeads are solid tiny spherical plastic that are less than one millimeter in dimension. They are used in exfoliating personal care products, cosmetics and toothpastes. They are typically made of polyethylene or

polypropylene, polyethylene terephthalate or nylon. Traditionally, natural exfoliators or cleansing substances such as grounded almonds, salt, or oatmeal were used, but about 50 years ago they are now gradually replaced by plastic personal care products. Surprisingly, negative consequences of the prolong use of plastic products in the past few years were revealed. Small pieces of plastics (usually acrylic, melamine, or polyester) are blasted at a high pressure at machines, engines, or ship hull to get rid of paint or rust. They are reused until they are no longer effective and eventually can become contaminated with heavy metals. Microplastics are also used in biomedical research applications. Abiotic factors such as waves, sunlight, or other physical stresses causes weathering of larger plastic materials originated from “Garbage Patches” or untreated waste in turn releasing small particles into the environment. In recent study it was found that about 15% to 31% are primary micro plastics consisting of synthetic fibers (synthetic rubber, laundry). Negative impact of microplastics arises when they escape through the water filtration process and eventually make it out into rivers and oceans due to their micro size. Consequences of micro plastics in ocean and other water bodies are horrifying.

Aquatic life such as fish, worms, zooplankton, crustaceans and other aquatic animals assume the microplastics as false food particles causing severe lethal health problems. Fish larvae need small feed depending on the smaller mouth size, and also, for this type of larvae the stomach is not fully developed and they obtain digestive enzymes from the live feed they prey upon. Another advantage of live feed is that fish larvae prefer moving feed rather than inert feed during early stages of development. As copepod is important component of trophic food web and as promising source of essential nutrition to fish and other cultivable organism, the present study aimed to made attempt on the effect of microplastics ingestion on biochemical composition of marine copepod *O. dissimilis*.

Materials and methods

The copepod *O. dissimilis* culture and microplastics ingestion experiment in copepod *Oithona dissimilis* were done according to the standard procedure as explained in Chapter IV under materials and methods part. The control and microplastics exposed copepod *O. dissimilis* were harvested from the respective containers and subjected to the estimation of moisture, protein, carbohydrate, lipid, ash, amino acids and fatty acids as followed the standard procedures to know the impact of microplastics ingestion on biochemical profile of copepod *O. dissimilis*.

Estimation of Moisture

1 g of control and tested copepod sample was taken and the excess moisture was removed using a filter paper (Rajendran, 1973). Then the sample was set to dry in a hot air oven at a constant temperature of 60°C till the wet sample was dried thoroughly. Then the moisture content of the sample was calculated by subtracting the dry weight of the sample from the wet weight of the sample. The percentage of moisture content was calculated using the following formula:

$$\text{Moisture \%} = \frac{\text{Wet weight of sample} - \text{Dry weight of sample}}{\text{Wet weight of the sample}} \times 100$$

Estimation of Protein

The percentage of protein content in the control and experimental copepod was estimated by following the Biuret method described by Lowry *et al.* (1951). 100 mg of copepod sample was homogenized with double distilled water and the extract was centrifuged at 4000 rpm for 10 minutes. To 1ml of supernatant, 4ml of Biuret reagent was added and incubated for 20 minutes. The optical density (OD) of the colour developed was

read at 540 nm using spectrophotometer and the protein was calculated by referring the standard graph of Bovine Serum Albumin. The result was expressed in dry weight.

$$\text{Protein (\%)} = \frac{\text{OD of sample} \times \text{standard value} \times \text{total volume}}{\text{Weight of sample} \times \text{Volume of extract}} \times 100$$

Estimation of Carbohydrate

Carbohydrate was estimated according to the procedure of Dubois *et al.* (1956). Copepod sample (25 mg) was homogenized with double distilled water and centrifuged: 1ml of 5% phenol solution and 5ml of concentrated sulphuric acid were added and it was allowed to react for 30 minutes and then the OD value was measured at 490 nm using UV–Spectrophotometer. The standard value was obtained by using glucose and the carbohydrate percentage was then calculated.

$$\text{Carbohydrate (\%)} = \frac{\text{Standard value} \times \text{OD of the sample}}{\text{Weight of the sample taken}} \times 100$$

Estimation of Lipid

For the estimation of lipid, chloroform: methanol method was followed (Folch *et al.*, 1956). Copepod sample (400 mg) was homogenized with 5ml of chloroform: methanol mixture and filtered by a fat filtering unit. The filtered solution was poured into a previously weighed 10 ml beaker and kept in an oven at 700°C for 24 hrs. The difference in weight between the empty beaker and the beaker containing fat was expressed as the amount of fat in the sample analyzed.

$$\text{Lipid (\%)} = \frac{\text{Amount of lipid in the sample}}{\text{Weight of the sample taken}} \times 100$$

Estimation of Ash

The ash content was determined by burning oven dried copepod sample in a muffle furnace at 550 °C according to the standard procedure of AOAC (1995). The percentage of ash content was determined by using the following formula:

$$\text{Ash (\%)} = \frac{\text{Ash content}}{\text{Dry weight}} \times 100$$

Analysis of amino acids

Sample digestion

A 100 mg of copepod samples were weighed in an electronic balance and transferred to labeled glass test tubes. 1 ml of 6M Hydrochloric acid solution was added with the sample in specified test tubes. These test tubes were sealed at the top under vacuum by high-temperature gas flame, conducted triplicates of samples. All the sealed tubes were kept in a hot-air oven at 110 °C for 48 hours continuously.

Test solution preparation

After completion of digestion, broken the tubes at the top and transferred the digest into glass beaker, rinsed the tubes 5 times with distilled water. The acid in the digest was evaporated to core dry under vacuum using the Rotary vacuum evaporator. The residual content was dissolved with distilled water and made up to 2.4 ml in a centrifuge tube. This solution contains 41.6 µg raw sample in 1µl distilled water and used as a test solution for amino acid profile analysis by the HPTLC technique.

Sample and Standard amino acid loading

1 µl of each test solutions were loaded as 5 mm band in pre-coated Silica gel 60 F254 TLC plate (10cm x 10cm) using 100 µl Hamilton syringe and CAMAG-LINOMAT 5 instrument. 1 µl of each Group I, II, III and IV standards were loaded in the plate for analysis as separate tracks.

Spot development

The samples loaded plate was kept in TLC twin trough developing chamber with respective mobile phase (Amino acids), 20 minutes for chamber saturation. After chamber saturation, the plate was developed in respective mobile phases up to 90 mm.

Photo-documentation

The developed plate was dried by hot air oven to evaporate solvents from the plate. The plate was documented using a photo-documentation chamber (CAMAG-REPROSTAR 3) at visible light, UV 254 nm and UV 366 nm mode.

Derivatization

The plate was sprayed with respective spray reagent (Amino acids) and dried at 100°C in Hot air oven. After derivatization, the plate was documented at visible light, UV 366 nm and UV 254 nm using CAMAG-REPROSTAR 3.

Scanning

The plate was fixed in the scanner stage and scanned at 500 nm using CAMAG-TLC SCANNER 3. The R_F value and Peak area of each track were observed for the quantification study. The software used was to win the CATS 1.3.4 version.

Calculations

Sample concentration : 100 mg of raw material in 2.4 ml distilled water

The loaded volume of test solution : 1 µl (41.6 µg of raw material)

Individual Amino acid content in % : $\text{Conc. of amino acid in } \mu\text{g} / 41.6 \mu\text{g} \times 100$

Fatty acids analysis of the *O. dissimilis*

The methanolic extract of *O. dissimilis* underwent gas chromatography-mass spectrometry (GC-MS) analysis (GC-MS - QP-2010 Plus, Shimadzu, Tokyo, Japan) with

the thermal desorption (TD) system 20. Experimental conditions of the GC-MS system were as follows: Trace-5 mass spectrometry capillary standard non-polar column, dimension: 30 meters; internal diameter: 0.25 mm; film thickness: 0.25 μm . The flow rate of the mobile phase (carrier gas: helium) was set at 1.2 ml/min. In the gas chromatography phase, the temperature programme (oven temperature) was 80°C, which was raised to 250°C at 10°C/min, and the injection volume was 1 μl . Samples dissolved in chloroform were run fully at a range of 50-650 mass-to-charge ratio (m/z) and the results were compared by using the Wiley Spectral Library Search Programme ([http:// www.sisweb.com/software/ms/wiley-search.html](http://www.sisweb.com/software/ms/wiley-search.html)).

Results

Effect of MPs on moisture content of *O. dissimilis*

The percentage of moisture content was high (83.12%) in control copepod than the microplastics exposed copepods. The lowest moisture content was recorded in all MPs exposed copepods 0.1. MPs exposed copepod μm showed low moisture (68.77%) content followed by 0.5 μm MPs ingested copepod (73.7%) and 2 μm MPs exposed copepod (69.13%) (Fig. 36).

Effect of MPs on protein content of *O. dissimilis*

In the present study, maximum protein content (63.07%) was noticed in control copepod, whereas in MPs exposed copepods it was 43.20%, 46.5% and 51.50% in 2.0 μm , 0.1 μm and 0.5 μm MPs exposed copepod respectively (Fig. 36).

Effect of MPs on carbohydrate content of *O. dissimilis*

The percentage of carbohydrate (10.92 %) was found to be high in control copepod, whereas it was low in MPs ingested copepods. The order of carbohydrate content in the MPs

ingested copepod was as follows: 0.1 μm 0.5 μm and 2.0 μm noticed as 10.33%, 8.93% and 9.57% respectively (Fig. 36).

Effect of MPs on lipid production of *O. dissimilis*

In the present investigation control copepod showed highest lipid content of 13.33% when compared to MPs exposed copepod. The lowest lipid concentration of 8.97% was noticed in 0.1 μm MPs ingested copepod followed by 0.5 μm MPs exposed copepod (10.7%) and 2.0 μm MPs ingested copepod (11.21%) (Fig. 36).

Effect of MPs ingestion on Ash content of *O. dissimilis*

The ash content was higher (4.21%) in control group whereas it was lower in 0.1 μm MPs ingested group (3.53%) followed by 0.5 μm MPs exposed copepod (3.93%) and 2.0 μm MPs ingested copepod (4.17%) (Fig. 36)

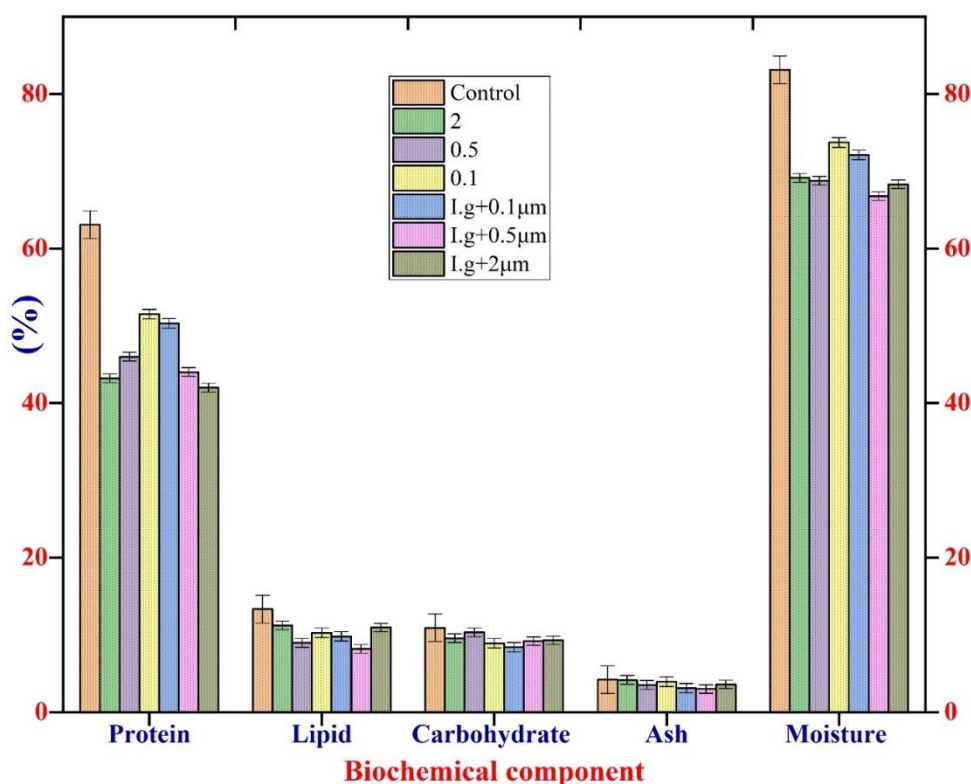


Fig. 43. Effect of MPs ingestion on biochemical composition of copepod. *O. dissimilis*

Effect of MPs on amino acids content of *O. dissimilis*

Amino acid composition in the control and treated marine copepod of *O. dissimilis* revealed 11 nonessential amino acids (NEAA) and 9 essential amino acids (EAA). The NEAA such as aspartic acid, glutamic acid, asparagine, serine, gultamine, glycine, arginine, alanine, cystine, tyrosine, proline and histidine were found to be low in the MPs ingested copepod (2, 0.5 and 0.1 μm) compared to control copepod and EAA such as histidine, valine, methionine, iso-leucine, phenyl alanine, leucine, lysine, tryptophan, and threonine were showed low in the MPs exposed copepod (2, 0.5 and 0.1 μm) when compared to control copepod (Table. 20).

Amino acids		Control (mg)	MPs (0.1 μm) (mg)	MPs (0.5 μm) (mg)	MPs (2 μm) (mg)
NEAA	Aspartic Acid	403.3	314.4	245.5	194.4
	Glutamic Acid	229.5	193.5	112.3	93.5
	Asparagine	225.6	209.4	195.8	19.3
	Serine	93.5	88.7	98.3	83.5
	Gultamine	225.3	215.4	199.5	193.4
	Glycine	306.7	235.6	219.3	209.5
	Arginine	293.6	285.3	296.5	243.6
	Alanine	319.3	304.3	268.5	269.3
	Cystine	349.3	339.2	325.3	295.3
	Tyrosine	349.8	349.2	331.9	325.2
	Proline	304.3	285.6	219.3	293.5
EAA	Histidine	424	355.8	359.6	353.5
	Valine	95.3	99.3	94.3	83.5
	Methionine	13.4	9.9	9.6	9.4
	Iso-Leucine	205.2	198.3	178.4	139.5
	Phenyl Alanine	89.4	86.6	73.5	53.5
	Leucine	98.3	189.5	73.5	32.5
	Lysine	263.5	209.3	193.5	119.3
	Tryptophan	41.5	38.5	28.4	8.3
	Threonine	319.3	246.5	225.6	209.3

Table 20. Effect of MPs ingestion on amino acids profile of *O. dissimilis*

Effect of MPs on fatty acids composition of *O. dissimilis*

In the present study, a total of 6 fatty acids were quantified in the tissue of control and experiment marine copepod *O. dissimilis*. Of which, three were saturated fatty acids (SFA), one was monounsaturated fatty acids (MUFA), and two were polyunsaturated fatty acids (PUFA) (Table 21). The SFA, such as Palmitic acid, Margaric acid, and Stearic acid were notice low in *O. dissimilis* exposed to different size MPs (2, 0.5 and 0.1 μm). The MUFA such as Oleic acid was low in MPs exposed copepod and PUFA such as linolenic acid, alpha linolenic acid was recorded low in MPs ingested (Table. 21)

Fatty acids		Control	MPs (0.1 μm) (gm)	MPs (0.5 μm) (gm)	MPs (2 μm) (gm)
SFA	Palmitic acid (C16:0)	0.4934	0.1935	0.1615	0.1495
	Margaric acid (C17:0)	0.0083	0.0034	0.0039	0.0385
	Stearic acid (C18:0)	0.4936	0.2035	0.1683	0.1835
MUFA	Oleic acid (C18:1; n-9)	0.7953	0.3318	0.2704	0.2353
PUFA	Linolenic acid (C18:2; n-6)	0.6835	0.2986	0.2385	0.1978
	Alpha linolenic acid (18; n-3)	0.7936	0.3165	0.2083	0.1836

Table 21. Effect of MPs ingestion on fatty acids profile of copepod *O. dissimilis*

Discussion

The biochemical composition of the copepod *O. dissimilis* generally characterized by substantial amounts of protein, lipids, carbohydrate, ash, moisture, high levels of n-3 PUFA (particularly DHA and EPA). Furthermore, the biochemical composition of algae-fed *O. dissimilis* was significantly greater, but the biochemical composition of microplastic-exposed copepods decreased. Protein, lipid, ash, and moisture contents of control *O. dissimilis* were found to be greater than those of microplastic-exposed copepods in this experiment. The biochemical composition of cultivated copepods and other live feeds has been examined by several authors. Santhanam and Perumal (2012) reported greater protein

content in the cyclopoid copepod *Oithona rigida* while Rajkumar and Vasagam (2006) recorded higher protein content in the calanoid copepod *Acartia clausi*. Protein is the predominant biochemical component of copepods, as demonstrated in this study and agreed upon by previous researchers (Ananth, 2015; Nandakumar, 2014).

It is well known that the basic compounds like protein and carbohydrate to high level of amino acids and fatty acids in copepods is essential for the better permissive of the organic production and biogeochemical cycle of cellular elements of organisms in the oceanic and estuarine ecosystem. The result of the present study indicate that significant biochemical component was noticed in copepod, *O. dsimilis* (control). Similar reports were stated by Nageswara Rao and Krupanidhi (2001); Ashok Prabu *et al.*, (2005); Rajkumar *et al.*, (2008). In copepods concentration of carbohydrate was very low when compared to other biochemical compounds such as protein and lipid. Similar findings were reported earlier by many workers (Maruthanayagam and Subramanian, 1999; Nageswara Rao and Krupanidhi, 2001; Ashok Prabu *et al.*, 2005; Rajkumar *et al.*, 2008).

In the present study, low carbohydrate content was noticed which may be due to glycogen process (storage carbohydrate). Besides, the utilization of carbohydrate glucosamine during the chitin synthesis in crustaceans may prone to the decrease of carbohydrate level in copepods (Ashok Prabu *et al.*, 2005). Goswami *et al.* (2000) reported that carbohydrate content of zooplankton community is dependent upon its composition, declining in gelatinous forms than those with calcareous shells and increasing with copepods. The fluctuations in glycogen content of animals generally depend upon their feeding activities (Nageswara Rao and Krupanidhi, 2001). The low carbohydrate content and high levels of protein in zooplankton suggest that protein, in addition to lipid, may function as a food reserve (Ashok Prabu *et al.*, 2005). It is similar to the values observed by Krishnakumari and Goswami (1993) in wild copepods, while more recently, Ananth and

Santhanam (2011) and Nandakumar (2014) discovered that protein content in cultivated copepods such as *Macrosetella gracilis* and *Nitocra affinis* was higher.

The protein content was high in control copepod whereas microplastic ingested copepods showed low protein content. Protein content are the major compounds which, act as an energy reserve. The deviations in the protein content might be due to the utilization of metabolic substrate (Nageswara Rao and Krupanidhi, 2001).

The lipid content was high in control and low in treatment group. The variation in the lipid content might be due to type of food fed by the organisms. The result was supported by findings of Ashok Prabu *et al.* (2005) who observed the variations in the lipid content of copepod from marine environment. Nageswara Rao and Krupanidhi (2001) also noticed some variations in the lipid content which can be attributed by its storage and utilization during periods when it serves as an effective energy reserve.

The lipid content variation among the live feeds might be due to the difference in the food of availability and water quality parameters (Ashok Prabu *et al.*, 2005). The variation in lipid content among live feeds, according to Nageshwara Rao and Krupanidhi (2001), might be linked to its storage and usage during feed-free conditions when it is used as an effective energy reserve. The carbohydrate content of algae-fed copepods was greater than that of copepods exposed to microplastics. Low carbohydrate content, of copepod have been studied by several researchers (Santhanam and Perumal, 2012; Jeyaraj, 2012; Ananth, 2015).

The present study reported that amino acids like aspartic acid, cystine, thyrosine, glutamic acid, glycine, and proline in *O. dissimilis* which are most abundant agreed with pioneer research works (Santhanam, 2002; Rajkumar, Santhanam and Perumal, 2004; Ashok Prabu, Perumal and Rajkumar, 2005; Perumal *et al.* 2009). The low amino acids

reported in MPs exposed copepod might be due to the adverse effect of microplastics caused in the metabolism of copepod. The essential and non-essential amino acids composition of microplastic-exposed *O. dissimilis* was lower, and the relative amount was higher in algae-fed copepods (Yurkowski & Tabachek, 1979; Watanabe *et al.*, 1983; Kibria *et al.*, 1999). (Yurkowski and Tabachek, 1979; Watanabe *et al.*, 1983; Kibria *et al.*, 1999). In general, amino acid profile of plankton is genetically programmed than diet related. In the present study, 17 amino acids was observed in Algae fed copepod and microplastic exposed copepod. Only very limited information is available on the amino acid content of microplastic exposed copepods. Perumal *et al.* (2009) have reported 16 and 15 amino acids in wild copepods, *A. spinicauda* and *Oithona similis* respectively. Similarly, some foreign authors are also reported that the rich amino acids were observed in microalgae fed copepods (Van der Meeren, Olsen, Hamre and Fyhn, 2008; Drillet, Jorgensen, Sorensen, Ramlov, and Hansen, 2006)

Fatty acids are essential for the structural and functional integrity of the cell membranes of copepods. Zooplankton includes significant quantities of arachidonic acid, which supports in the development and survival of larvae (1995). The fatty acids such as MUFA, SFA, and PUFA were found to be higher in algae fed copepods, however microplastic exposed copepods had a lower level of fatty acid composition, which might be related to the presence of microplastic granules inside the copepod which caused anti cell membrane activity (Cole *et al.* (2019). The impact of microplastics on the biochemical and nutritional characteristics of *O. dissimilis*, were investigated in this study. As a result, these indicators should be used in future research to evaluate the quality of food resources affected by plastic trash or other contaminants.

In general the biochemical composition was high in control group while it was low in treatment groups. The microplastic ingested copepod showed variations in the nutritional

profile. It might be due to the interference of microplastic amalgams to the biochemical structure of the organism which shows variance in the nutritional profile. Since it is a first hand research work on analysis of the impact of microplastics ingestion on the nutritional profile of copepods a very few limited sources of information are available for interpretation. In future, advance studies are needed along this baseline which is important for the understanding their physiological functions, metabolism and nutritive value of copepods with reference to microplastics ingestion.

Summary & Conclusion

10. Summary and Conclusion

The present study comprises five different chapters namely 1) Ecology of marine copepods. 2) Morphological and molecular identification of marine copepod, 3) Optimization of culture conditions for copepod *O. dissimilis*, 4) Impact of microplastics ingestion on physiology of marine copepod *Oithona dissimilis* and 5) Impact of microplastics ingestion on nutritional profile of selected marine copepod *O. dissimilis*. In chapter-I, Spatial and temporal variations of physico-chemical parameters and biological parameters observed which pointed those remarkable seasonal variations. Four seasons (summer, premonsoon, monsoon and post monsoon) were characterized by different physico-chemical and biological properties. The result of the present study indicated that the rainfall showed significant changes on physico-chemical characteristics of the Nagapattinam coastal waters. The pH of the Nagapattinam coastal water was higher during summer season might be due to more phytoplankton production while low pH noticed in monsoon season might be due to fresh water input and less phytoplankton production. As like temperature and salinity, pH also positively supports for the high copepods density and diversity. It is clearly indicated that the temperature and salinity were significantly enhanced the copepod density and species diversity from Nagapattinam coastal waters. Totally 38 species of copepods were identified from Nagapattinam coastal waters. Of these 27 species were observed which belong to Calanoida, 7 Cyclopoida, and 4 Harpacticoida. The population density of copepod was recorded higher in station 2 (Sea mouth) and 3 (Estuary) might be due to favorable physico-chemical conditions prevailed. The diversity of copepod was found higher in station 1 (Neritic Zone) that is sea might be due to occurrence of more neritic and oceanic species dominance. During the one-year study, copepod species such as *Acartia spinicauda*, *Pontella fera*, *Pontella dane*, *Centropages furcatus*, *Acrocalanus*

gracilis, *Labidocera minuta*, *Labidocera acuta*, *Temora turbinata*, *Pseudodiaptomus aurivilli*, *Calanopia* sp., *Euterpina acutifrons*, *Nitocra affinis* and *Dioithona rigida* were recorded in all the seasons in appreciable numbers which might be due to their high reproduction potential and adaptability to the changing environmental conditions.

Chapter-II, it can be concluded that the detailed descriptions of *O. dissimilis* will certainly help in solving the taxonomic problem surrounding the genus. However, the COI more slowly evolving nuclear small subunit gene has used to resolve deep phylogenetic relationship among the species within the family levels including invertebrates. Thus, in the present result the COI for *O. dissimilis* showed closest similarity from the available homologous sequence in the NCBI database and which showed deep phylogenetic relationship within family level. The sequence of *O. dissimilis* submitted in the GenBank would provide a reference sequence for the identification of the *O. dissimilis* population studies in the future.

Chapter-III, the optimization of the copepod *O. dissimilis* for laboratory culture was carried out using different environmental parameters. Among the elected parameters tested *O. dissimilis* resulted higher survival, nauplii production and population density in 28°C temperature, 500 lux light intensity, 25 PSU salinity and 8 pH. The microalgae *I. galbana* with a concentration of 30,000 cells/ml was proved to be a better feed option for *O. dissimilis*. The developmental period was shorter in copepods fed with *I. galbana*. The superiority in nutritional value and the smaller cell size of *I. galbana* makes it a suitable mono algal feed for culturing the copepod *O. dissimilis*.

Chapter IV and V, the results on MP's ingestion experiment reveals that the survival, nauplii production and population growth of copepod *O. dissimilis* was adversely affected. The present investigation clearly indicate that the MPs ingestion drastically reduce the ash,

moisture, protein, carbohydrate and lipid content in copepod *O. dissimilis*. Likewise, the microplastics ingestion was significantly decrease the amino acids content in copepod (*O. dissimilis*) might be due to unfavorable physiological and biochemical metabolism of the organism. The significant decrease in fatty acids content suggest that the MPs had adverse effect on the production of fatty acids in copepod *O. dissimilis*. The present findings strongly insists that the adverse impact of microplastics on physiology and biochemistry of copepod may affect the production of commercial important group of organisms such as fin fishes and shellfishes as they are rely on copepods as their main diets. Further, attempt should be made on the concomitant effect of microplastic ingestion in marine food chain or food web.

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Impact of polystyrene microplastics on major marine primary (phytoplankton) and secondary producers (copepod)

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Abstract

The effect of microplastic adsorption on marine microalgae *Tetraselmis suecica*, *Amphora subtropica*, and copepod *Pseudodiaptomus annandalei* was investigated in the present study. Fluorescence microscopic images were used to evaluate MP interactions with algae and copepods. *T. suecica* growth rate decreased with effects of 0.1 µm polystyrene exposure to 75 µl/100 ml (0.899 to 0.601 abs), 50 µl/100 ml (0.996 to 0.632 abs) and 25 µl/100 ml (0.996 to 0.632 abs), respectively. On the other hand, at 10th day of experiment, the control *T. suecica* showed the highest growth rate (0.965 abs), chlorophyll concentration (Chl-‘a’ = 21.36 µg/L; Chl-‘b’ = 13.65 µg/L), and cell density (3.3×10^6 cells/ml). A marine diatom *A. subtropica* absorbed 2.0 µm microplastics, and the maximal inhibition rate increased at higher MP concentration until 10th day. The highest MPs (75 µl/100 ml) treatment resulted in decreased growth rate of *A. subtropica* from 0.163 to 0.096 abs. *A. subtropica* (without MPs) had the highest lipid concentration of 27.15%, whereas *T. suecica* had the lowest lipid concentration of 11.2% (without MP). The maximum survival (80%) of *P. annandalei* was found in control on 15th day whereas on 12th day, the microplastics ingested copepod had the lowest survival rate (0%). On 15th day, the maximum Nauplii Production Rate (NPR) (19.33) female⁻¹ was observed in control, whereas the minimum (17.33) female⁻¹ NPR was observed in copepod ingested with MPs. The maximum lipid production (17.33% without MPs) was reported in control, whereas MPs fed copepods had the lowest lipid production (16%). Long-term exposure to polystyrene microplastics significantly reduced algae growth and chlorophyll concentration and also NPR and lipid concentration rate of copepod. We inferred that microplastic exposure of algae and copepods might results in persistent decreases in ingested carbon biomass over time.

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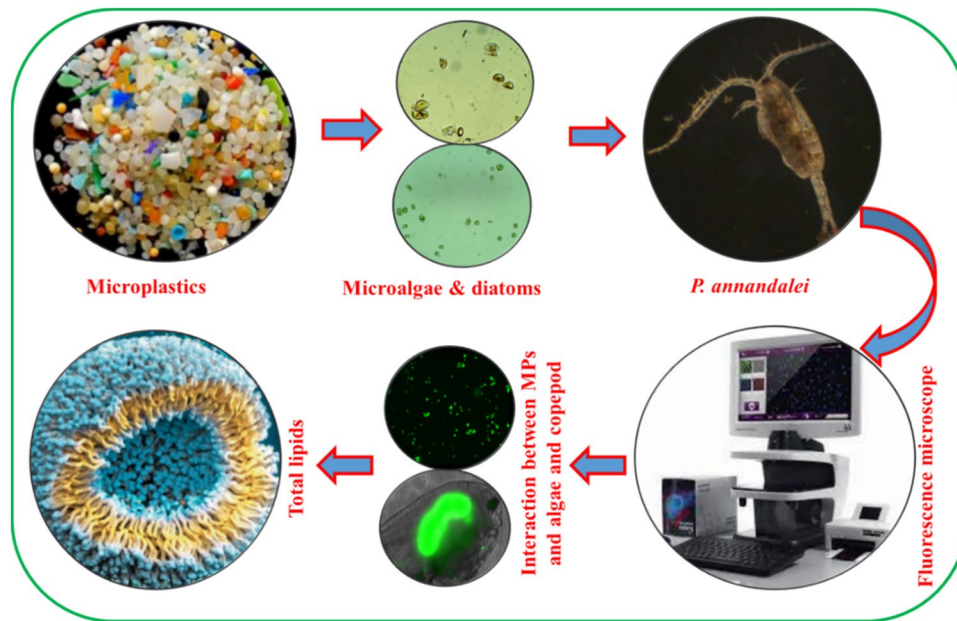
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Graphical abstract



Keywords Microalgae · *Pseudodiaptomus annandalei* · Microplastics · Diatom · Total lipids

Introduction

Plastic production has increased significantly since the large-scale industrial manufacturing started in early 1950s. Plastics are used in almost every aspects of daily life (Plastics Europe, 2016) and the majority of plastics are used in automotive industry (8.9%), electrical and electronic devices (5.8%), agriculture (3.3%), packaging (39.9%), building and construction (19.7%), and other sectors (22.4%). The plastic products would gradually break down into fragments over time, especially if they were continuously exposed to ultraviolet radiation (of sunlight) and high temperatures. This fragmentation would reduce the plastic material sizes from macroscopic to microscopic and finally nano-sizes. The increasing awareness of plastic pollution has resulted in the documentation of more scientific and societal concerns regarding the effects of microplastics (MPs) with diameter less than 5 mm (Gregory and Ryan, 1997; Kako et al. 2011, 2014; Yoon et al. 2010; Kubota, 1994; Zarfl and Matthies, 2010; Isobe et al. 2014; Maximenko et al. 2012). Microplastics can be categorized as either primary or secondary MPs based on their manufacturing process. Primary microplastics are tiny particles that are released into the environment directly or indirectly through domestic and industrial effluents, spills and sewage discharge. The most commonly found primary microplastics are fibres, pellets, film,

fragments and spheres (Kang et al. 2015; Lusher 2015; Li et al. 2016). Secondary microplastics are produced by photo-oxidation of UV radiation, mechanical transformation and microbial degradation (Andrady and Neal, 2009; Cole et al. 2011). The smaller particles broken down into nanoplastics (1–100 nm) with toxicological characteristics (Koelmans et al. 2015; da Costa et al. 2016). Ingestion of microplastics by marine organisms including plankton, fish, benthic organisms that can be trophically transferred (Browne et al. 2008; Wright et al. 2013). Overall reported the impacts of microplastics on marine organisms such as growth delay, oxidative stress, reduction of feeding activity, genotoxicity, neurotoxicity and reduction of reproductive fitness (Mazurais et al. 2015; Li et al. 2018). Microplastics adsorption by microalgae influence the sinking rate of both microalgae and buoyant microplastics (Ballent et al. 2013). Biofouling of microplastics may also enhance their absorption by biota (Andrady 2011). Under stressful conditions such as a lack of light or nutritional shortage, microalgae produce polysaccharides molecules, known as exopolysaccharides which can subsequently coagulate, allowing algal cells to aggregate (Long et al. 2015; Staats et al. 2000). Copepods are secondary producer and popular model for ecotoxicological studies among zooplankton because they play an important role in aquatic ecosystem and are sensitive to environmental stressors (Ananth and Santhanam, 2011). Polystyrene is a transparent

polymer contains styrene monomers with specific gravity of 1.04–1.07 g cm⁻³ and its soluble in various organic solvents such as esters, aromatic hydrocarbons and ketones. PS is resistant to salts, alkalis, mineral oils, organic acids and alcohols. Less weight PS foam provides many applications such as roofing, refrigerators, building walls and freezers. PS caused the gastrointestinal tract infections and toxic to the cellular level (Cole et al. 2015). According to Cai et al (2018), PS particles from laboratories, might be a source of main plastics particulate pollutants and their particles can create a variety of issues depending on their size, shape and functional groups. Polystyrene is a primary particle caused the delayed growth, feeding capacity and reproductive development in marine organisms (Alimi et al., 2018). The aggregation and attachment of polystyrene to marine organisms from microalgae to copepod were not investigated by earlier studies; hence, the present study was aimed to assess the effects of microplastics adsorption on marine microalgae *Tetraselmis suecica*, *Amphora subtropica* with effects on growth rate, chlorophyll concentration, lipid profile besides survival, nauplii hatching and lipid profile of copepod *P. annandalei*.

Materials and methods

Experimental setup

Pre-cultured *T. suecica* (PSBDU002) and *A. subtropica* (KM099276) at the logarithmic growth phase were taken from microalgae culture collection of marine planktonology and aquaculture laboratory of Department of Marine science, Bharathidasan University, and added into 100 ml of Conway's and TMRL-enriched seawater medium was used as a control, of which 10% inoculum was added. Depending on the size of the microalgae, two distinct Polystyrene microplastics (0.1 µm (Lot# MKCH2797) amine modified fluorescent orange beads for *T. suecica* and 2.0 µm (Lot#MKCJ4402) fluorescent yellow green beads for *A. subtropica*) were obtained from Sigma-Aldrich and individually injected to each algal culture to achieve concentrations of 25, 50 and 75 µl/100 ml, respectively. All the experiments were incubated under controlled laboratory conditions that remained constant throughout the investigation (23 ± 1 °C, 50 µmol photons/(m² sec⁻¹) and a 12 h/12 h (light/dark) cycle). The aggregation and attachment of MPs to algae were evaluated over an entire culture cycle, from seeding to stationary growth phase for each algal species. The microalgal physiology was examined through the analysis of their growth rates, cell density and chlorophyll for assessing the photosynthetic ability which is used to identify the potential impacts of micro-PS on phytoplankton community. All groups were maintained as triplicate.

Determination of photosynthetic pigment

Chlorophyll was extracted by taking 0.5 g of algae to which 5 ml 90% acetone was added. It was kept undisturbed overnight and the optical density of the chlorophyll was measured next day with a UV/Vis spectrophotometer (Spectro 20D plus, U.S.A). The amount of chlorophyll was calculated according to the equations of Jeffrey and Humphrey (1975).

Chlorophyll analysis for green algae

Chlorophyll 'a' (µg/ml) = 11.93 E₆₆₄ - 1.93 E₆₄₇.

Chlorophyll 'b' (µg/ml) = 20.36 E₆₄₇ - 5.50 E₆₆₄.

Chlorophyll analysis for diatom

Chlorophyll 'a' (µg/ml) = 11.47 E₆₆₄ - 0.40 E₆₃₀.

Chlorophyll 'c' (µg/ml) = 24.36 E₆₃₀ - 3.73 E₆₆₄.

Determination of microalgae growth rate

Algal growth was measured by taking absorbance. Absorbance was measured every 2 days once for the period of 10 days by recording the changes in optical density at 680 nm with a UV/Vis spectrophotometer according to Lichtenthaler (1987).

Determination of algal cell density

Microalgal cell concentration was determined through cell counting using haemocytometer. The haemocytometer is a slide glass size 30 mm × 70 mm × 4 mm thick. It has 2 chambers and cells counts were performed at the center part. The specific growth rate of microalgae was calculated using Eq. 1 derives formula from Andersen (2005).

$$\text{Cell Density} = \frac{\text{Average number of cells per square} \times \text{Dilution factor}}{\text{Volume of square (10}^4\text{)}}.$$

Extraction of total lipids from microalgae

The total lipids of the algae were extracted by mixing chloroform-methanol (4:2 v/v) by adopting the standard procedure of Folch et al. (1957). A mixture of 2 ml methanol and 1 ml chloroform was made and added to 1 g algal biomass. It was kept for 24 h at room temperature to dissolve the lipids properly. The mixture was centrifuged at 3000 rpm for 10 min. Supernatant was separated, 2 ml of chloroform was again added to the pellets and shaken properly. It was

again centrifuged at 3000 rpm for 5 min and supernatant was separated. After adding 2 ml of 1% KCL to the supernatant, two layers were formed. Lower layer was pipette out and weighed.

$$\text{Lipid content (\%)} = \text{wt. of lipid (g)} \times 100 / \text{wt. of algal sample (g)}.$$

Collection and identification of copepods

The zooplankton samples were collected from the Vettar estuary (10°46' N Lat. 79°12' E Lon.) using plankton net with 158 µm mesh. The collected samples were immediately transported to laboratory by providing with vigorous aeration using battery aerator. The zooplankton samples were thoroughly rinsed to reduce the contamination from other zooplankters. From the samples, *P. annandalei* was isolated with fine brush, needle and stempel pipette and identified under microscope using the key of Davis (1955); Kasturirangan (1963). Based on the keys provided by the authors, the species was confirmed for their taxonomy and used for culture.

Copepod culture

About 50 individuals of *P. annandalei* were isolated and stocked in 1000 ml beaker contained seawater. The marine microalga *Isochrysis galbana* at the concentration of 25,000 to 30,000 cells/ml. was given as feed for copepod *P. annandalei*. The water quality parameters, viz., temperature (23–26° C), salinity (28–30 PSU) and pH (7–8.5) were maintained at on optimized condition. The generation time of *P. annandalei* under optimal conditions was about 10–12 days and having 6 nauplii and 6 copepodite stages including the adult. Finally, the adult gravid female copepods were used to restart the stock culture.

Microplastic beads

The latex beads, carboxylate-modified polystyrene, fluorescent yellow-green were purchased from (Sigma-Aldrich, USA) L4530 (Lot # MKCF0821) aqueous suspension with 2.0 µm mean particle size was used for the present experiment.

Experimental setup

The copepod, *P. annandalei* was cultured in filtered seawater. The 2 µm green fluorescent microplastic beads were used for the experimental study. The control copepods were fed with *I. galbana* with the concentration of 25,000 cells/ml and 2 µl/100 ml MPs was ingested in the experimental copepod. Control and experimental groups were triplicated.

Survival rate, nauplii production rate and total lipid was determined in control and experimental copepod.

Effect of MPs ingestion on survival rate of *P. annandalei*

The survival rate of copepods was analyzed for the period of 15 days. 10 numbers of healthy *P. annandalei* were picked up from the stock culture by using fine brush and then transferred to a 100 ml beaker filled with sterile seawater and fed with *I. galbana* and inoculated with 2.0 µm fluorescence yellow green MPs. The number of live copepod specimens remaining in each chamber was recorded daily. Dead copepods were removed from treatments. The survival rates of copepods in respect to experimental and control treatments were measured by subtracting the final density of copepods from initial density of copepods.

Assessment of MP ingestion on nauplii hatching of *P. annandalei*

For determining the nauplii producing capacity of microplastic-ingested *P. annandalei* and control *P. annandalei* five individual female with viable egg sacs were stocked in a glass test tube containing 20 ml of filtered seawater. Copepod *P. annandalei* was stocked in the beaker filled with seawater and 2 µl of MPs. The copepod stocked with only microalgae *I. galbana* was control. The copepod was examined at regular intervals (every 2 h) 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.

Determination of total lipids from *P. annandalei* fed with MPs

The copepod *P. annandalei* was cultured with control (*I. galbana*) and fed with MPs was harvested using the 48 µm mesh. The harvested copepod sample was first washed with filtered seawater followed by distilled water. After removing the excess moisture using blotting paper copepod sample was subjected to drying in an oven. Lipid content of copepod was estimated by the method of Folch et al. (1957). In brief, a known amount of dried copepod sample was homogenized in 10 ml of chloroform–methanol mixture (2/1 v/v). The homogenate was centrifuged at 2000 rpm. The supernatant was washed with 0.9% saline solution (KCl) to remove the non-lipid contaminants and allowed to separate. The upper phase was discarded by siphoning. The lower phase was allowed to dry in a hot air oven and the weight was taken.

$$(\%) \text{ of Lipid} = \frac{\text{Amount of lipid in the sample} \times 100}{\text{Weight of the sample taken}}.$$

Statistical analyses

The statistical analysis of the obtained data was assessed with one-way analysis of variance (ANOVA) with help of SPSS by using Regression Analysis Test (DMRT).

Results

Effect of MPs adsorption on algal photosynthesis

The green algae *T. suecica* (0.1 µm MPs) were subjected to three different concentrations of microplastics (25, 50, and 75 µl/100 ml) and 2.0 µm of green fluorescent MPs were injected in to *A. subtropica* culture flask with three different concentrations (25, 50 and 75 µl/100 ml). The impacts on their photosynthesis and growth rate were monitored every 48 h for 10 days.

Effect of MPs (0.1 µm) on growth rate, cell density and chlorophyll concentration of *T. suecica*

The growth rate, cell density and chlorophyll concentration of *T. suecica* was increased in microplastics suspension culture and no homo-aggregates were formed from day 1 to day 4. In MPs exposure treatment homo-aggregation was observed from 6th day onwards. However, the rate of growth and chlorophyll content decreased from 8th day to 10th day under 75 µl/100 ml (0.899 to 0.601 abs) MPs exposure followed by 50 µl/100 ml (0.996 to 0.632 abs) and 25 µl/100 ml (0.792 to 0.52 abs). The control experiment showed maximum growth rate (0.965 abs), chlorophyll concentration (Chl-‘a’ = 21.36 µg/L; Chl-‘b’ = 13.65 µg/L) and cell density (3.3×10^6 cells/ml) on the 10th day of experiment. Only the microalgae (in suspension) were taken into account for the estimation of growth rate and chlorophyll (Fig. 1). *T. suecica* exposed to 0.1 µm polystyrene for different exposure time revealed a positive correlation and significantly affected chlorophyll ‘a’ ($R^2 = 0.94$; $P < 0.01$) and ‘b’ ($R^2 = 0.89$; $P < 0.02$) concentrations when compared to control ($R^2 = 0.59$; $P > 0.05$). At different exposure periods, the growth rate of *T. suecica* was not significantly different from the control ($R^2 = 0.34$; $P > 0.081$). Cell density was decreased in experimental algae as compared with control showed non-significance and reduced maximum cell density concentration ($R^2 = 0.140$; $P < 0.496$).

Effect of MPs (2.0 µm) on growth rate, cell density and chlorophyll concentration of *A. subtropica*

Three growth phases (the lag phase, the logarithmic phase and the stationary phase) of *A. subtropica* over a 10 days of incubation period were clearly distinguished in the control groups and algal growth rate, cell density and chlorophyll continuously increased from 0.012 to 0.596 abs, 4.02×10^4 to 2.102×10^5 cells/ml and Chl-a = 3.23 to 15.23 µg/L and Chl-b = 1.56 to 7.15 µg/L from initial day to 10th day, respectively. Then the algae was exposed to 2.0 µm microplastics and the maximum inhibition rate was recorded with increased MPs concentration until 10th day. In the present study, the highest concentration (75 µl/100 ml PS) of microplastic exposure treatment flask inhibit the cell density from 6th day to 10th day. Under 75 µl/100 ml MPs treatments growth rate decreased from 0.163 to 0.096 abs at the end of the study (as compared to the control). Similarly, the chlorophyll concentration was also reduced under highest MP exposure with homo aggregates starting from 6th day to 10th day (Fig. 2). 2.0 µm polystyrene-ingested *A. subtropica* with different exposure days are significantly affected the chlorophyll concentration ‘a’ & ‘b’ as compared with control ($R^2 = 0.98$; $P < 0.03$) and ($R^2 = 0.98$; $P < 0.01$). *A. subtropica* growth rate was not significantly different with control at different exposure days ($R^2 = 0.22$; $P < 0.454$). Maximum cell density concentration decreased in ingested one against control ($R^2 = 0.76$; $P < 0.496$).

Effect of MPs on lipid concentration of *T. suecica* and *A. subtropica*

While many microalgal strains were found to naturally have high lipid content. Lipid accumulation refers to increased concentration of lipids within the micro algae cells. In the present investigation the lipid concentration of *T. suecica* and *A. subtropica* was analyzed in relation to control (Without MPs) and different concentrations of MP exposed algae. In the present study, maximum lipid concentration of 27.15% was observed in control *A. subtropica* (without MPs) and the minimum (11.2%) was observed in *T. suecica* (Without MPs). Only minimal changes were observed in the lipid content MPs exposed microalgae (Fig. 3). Lipid concentration was significantly lower in Polystyrene-exposed *T. suecica* compared to control ($R^2 = 0.82$; $P < 0.04$). Similarly, when *A. subtropica* was exposed to 2.0 µm PS, its lipid level reduced in comparison to the control ($R^2 = 0.92$; $P < 0.003$).

Survival rate of *P. annandalei*

In the present study, the maximum survival rate was observed in copepod fed with *I. galbana* (80%) whereas microplastics ingested copepods were died at the 12th day of

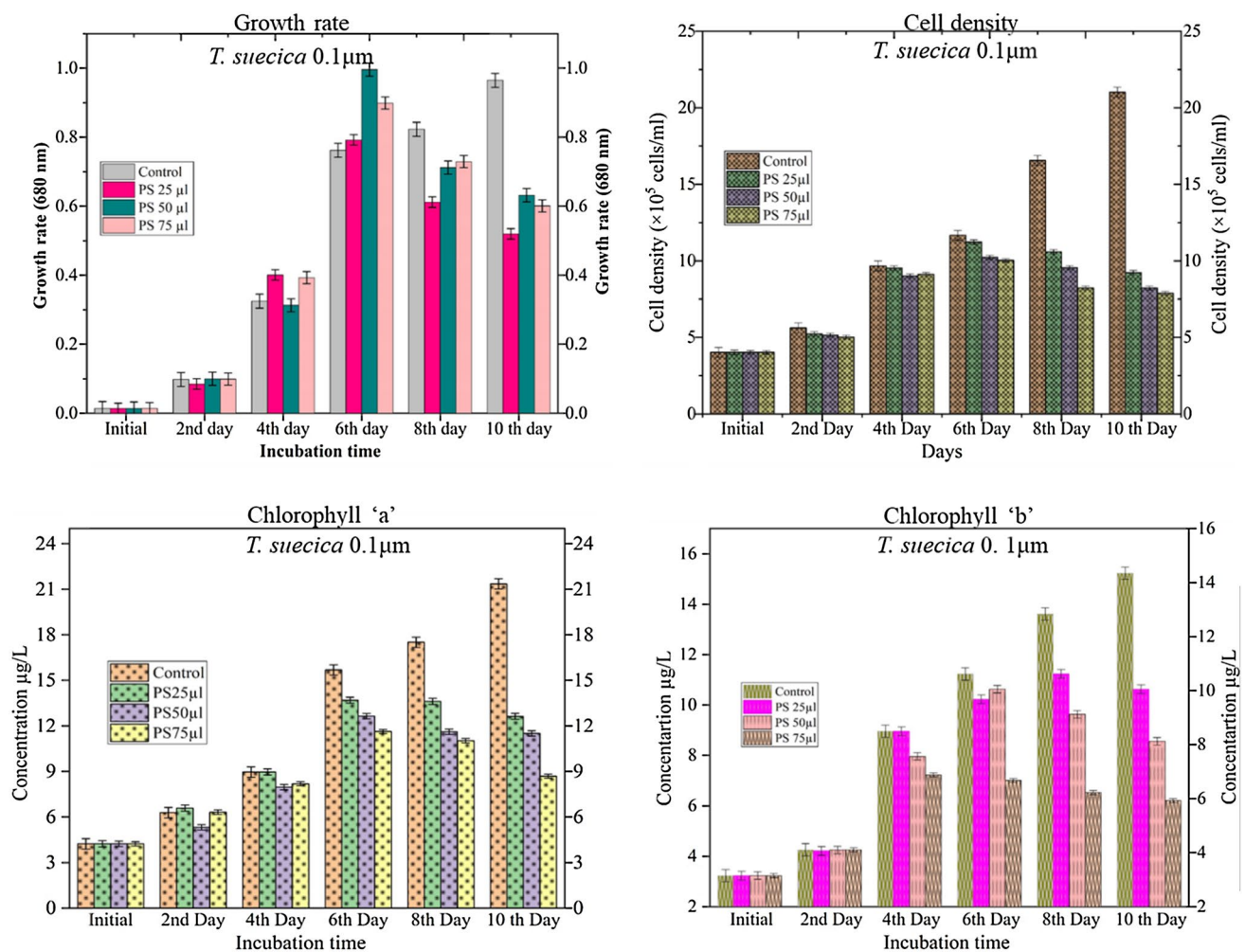


Fig. 1 Chlorophyll 'a' and 'b' concentration showed significance ($P < 0.01$; $P < 0.02$), cell density non-significance ($P < 0.496$) and growth rate not significance ($P > 0.081$), of *T. suecica* in response to MP exposure with size 0.1 µm

experiment and the survival rate of MPs ingested *P. annandalei* was 0% (Fig. 3).

Nauplii hatching rate of *P. annandalei*

The observed results revealed that the microplastic feeding adversely impacts the production of nauplii in copepods. The highest nauplii production rate was observed in the control copepod diet with an average of 19.33 nauplii female⁻¹ whereas the lowest nauplii production was observed in MPs ingested *P. annandalei* with 4 nauplii female⁻¹ (Fig. 3).

Lipids

In adult *P. annandalei*, approximately 40% of energy derived from their food goes toward the buildup of their lipid store. The presence of lipid concentration in copepod was based on the mass culture production. In the present investigation,

micro-algae fed copepod showed highest lipid production (17.3%) whereas the MPs ingested copepods result the lowest lipid profile (Fig. 3). Furthermore, we concluded that a feeding may result in an altered lipid profile. Yet, there was no difference in the total (Plate 1) lipid concentration of copepods at the end of the exposure period (Plate 2).

Discussion

Polystyrene (PS), a high-volume polymer with global market worth more than \$30 billion dollars, is one of the plastics often found in oceans (Transparency Market Research, 2014). Because marine microalgae are the major producers at the bottom of the food chain, there is concern about the potentially adverse effects of nano- and micro-sized plastic particles (Kaiser et al., 2011). According to Bhattacharya et al. (2010) the interactions between microplastics and

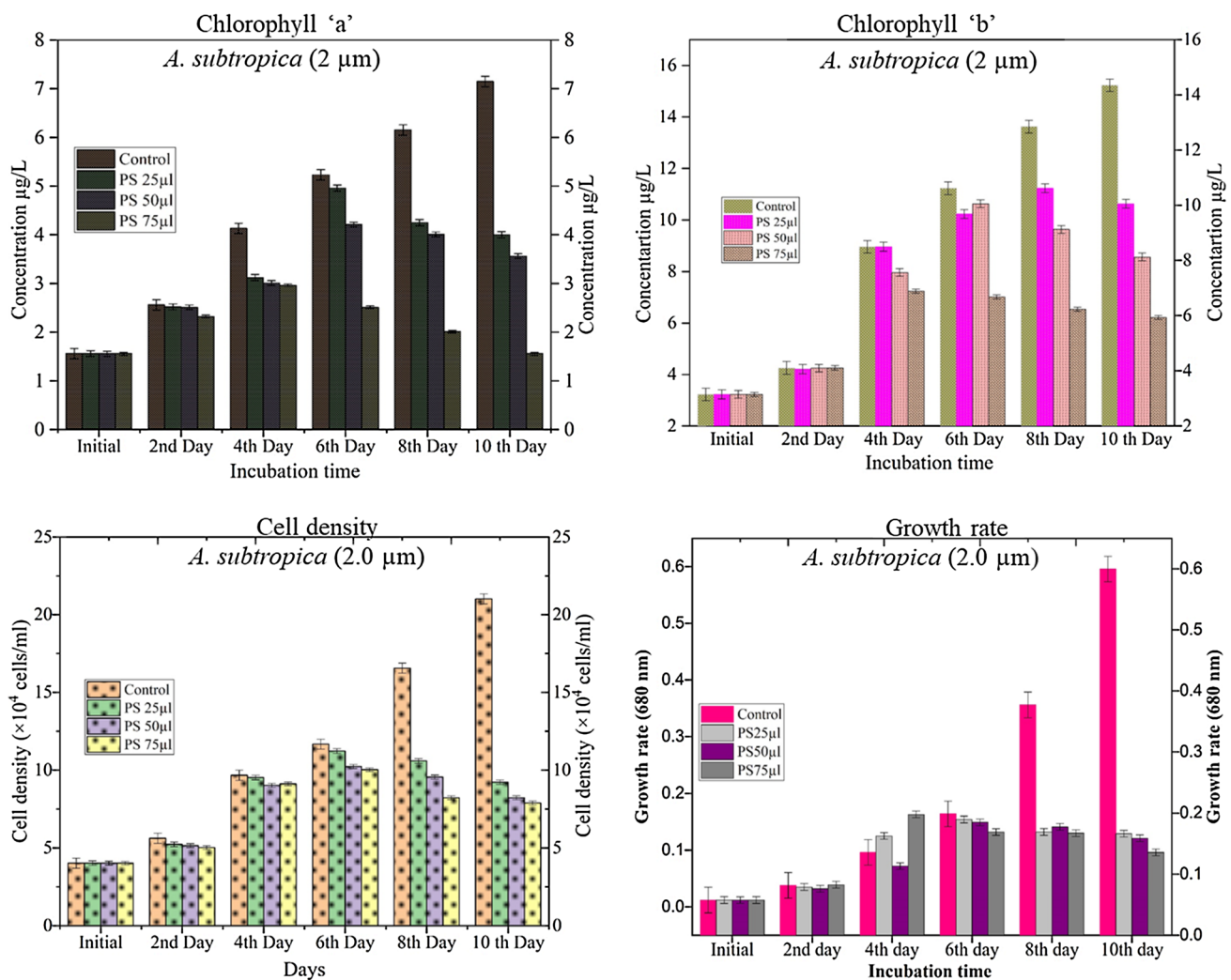


Fig. 2 Chlorophyll 'a' and 'b' concentration showed significance ($P < 0.03$; $P < 0.01$), cell density non-significance ($P < 0.496$) and growth rate not significantly ($P < 0.454$) of *A. subalpina* in response to MP exposure with size 2 µm

microalgae are expected to alter cell properties such as size and shape. Additionally, algal cell walls act as barriers to particle penetration and the cell wall characteristics may consequently influence particle sorption. Therefore, the present study was conducted in marine diatom, *A. subalpina* (with a silicate cell wall) and the marine alga *T. suecica* without a cell wall.

Microplastics debris, as an emerging environmental pollutant, is prevalent globally now. Therefore, it is crucial to have a basic understanding of the interactions between microplastics and the organisms. In the present study, the effects of microplastics on microalgae (*T. suecica*) was exposed to 0.1 µm size MPs and then the effects on their photosynthesis and growth were determined every 48 h for the period of 10 days. All the tests were performed under identical temperature, illumination and photoperiod conditions with the exponentially grown cultures at a start density

of (5×10^5 cells/ml) (allowing for exponential growth during the experiment). In contrast to photosynthesis, a clear effect of the MPs beads on the growth of *T. suecica* was observed. The highest MP concentration of 75 µl/100 ml showed the lowest microalgal photosynthesis, growth rate and cell density. The growth rate of *T. suecica* was higher on the 6th of the experiment, which might be owing to the homo-aggregation of microalgal cells. The reduction in cell density, algal photosynthesis and growth rate were exposed to 0.1 µm size with concentration of 25 µl/100 ml MPs beads was clearly reduced from 8 to 10th day when compared to the control.

When compared to other species, diatoms like *A. subalpina* expelled large amounts of sticky transparent exopolymeric particles, and these diatoms possess biogenic silica (Passow, 2002). *A. subalpina* is known to easily aggregate for these reasons. The influence of microplastics on the marine diatom *A. subalpina* was studied from the

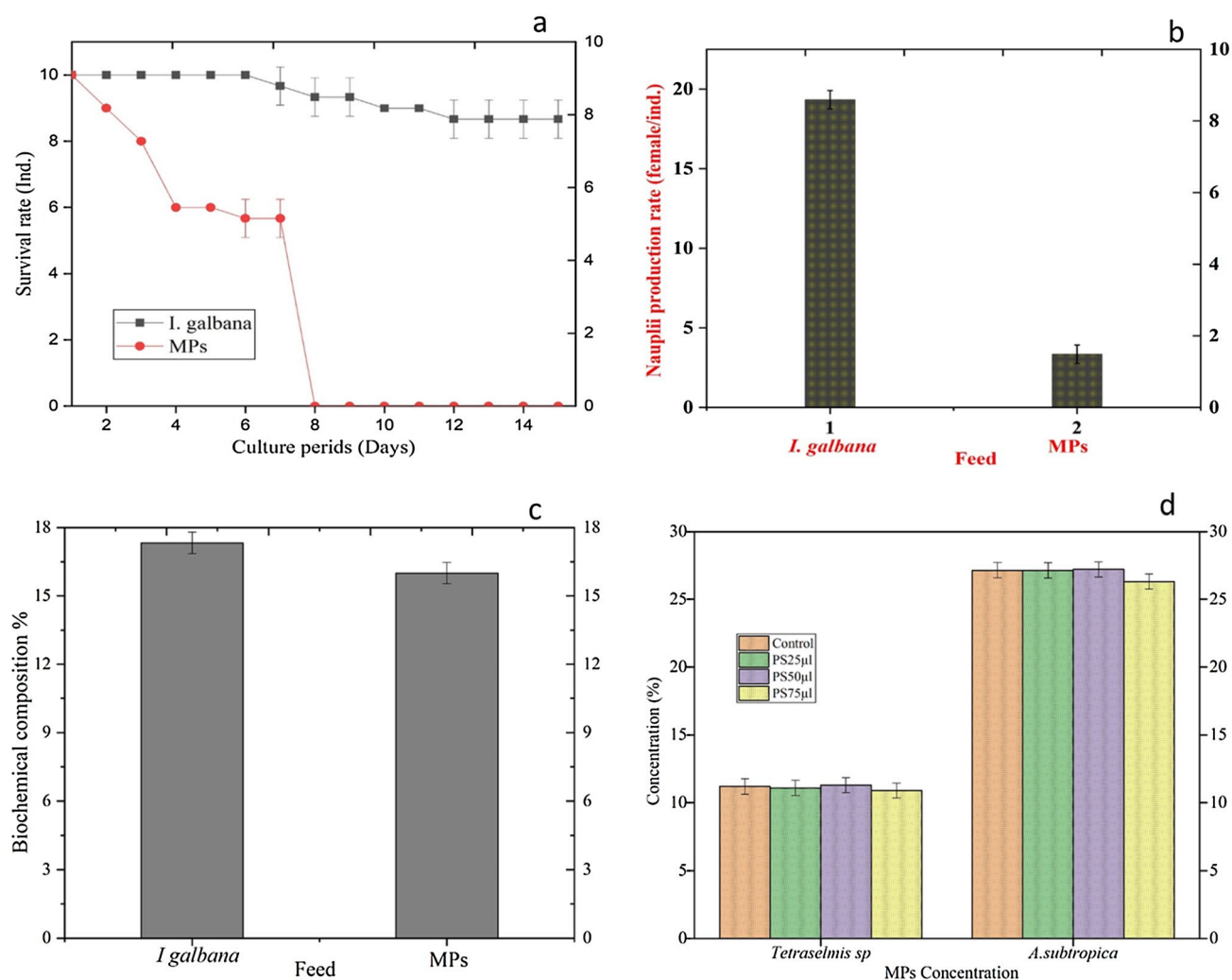


Fig. 3 Effect of MPs on survival rate (a), nauplii hatching rate (b), lipids of *P. annandalei* (c). The means values shown of three triplicates $\pm$ standard deviation and standard deviations were calculated

from replications and lipids of *T. suecica* and *A. subtropica* showed significantly ($P < 0.04$; $P < 0.003$) (d)

Plate 1 Fluorescence microscopic images of *T. suecica* and *A. subtropica* exposed to (0.1 and 2 μ m) MPs

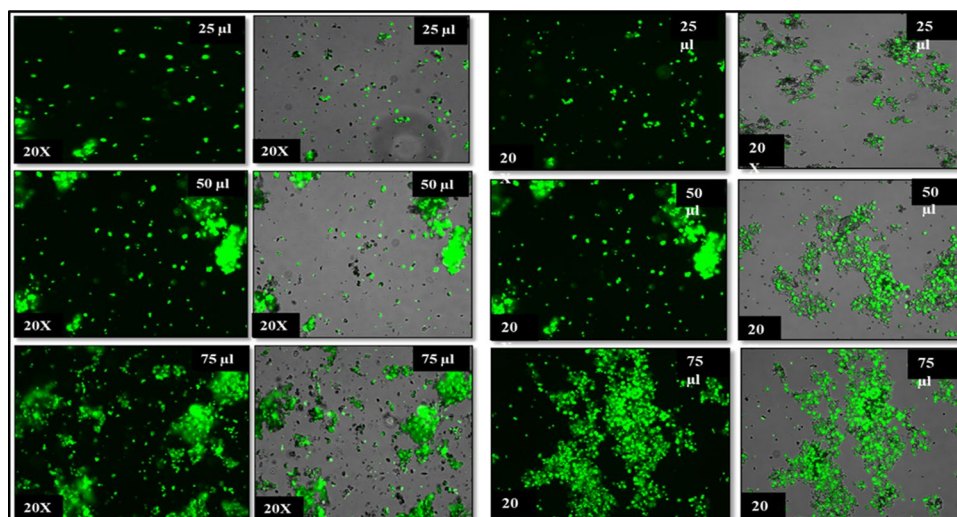
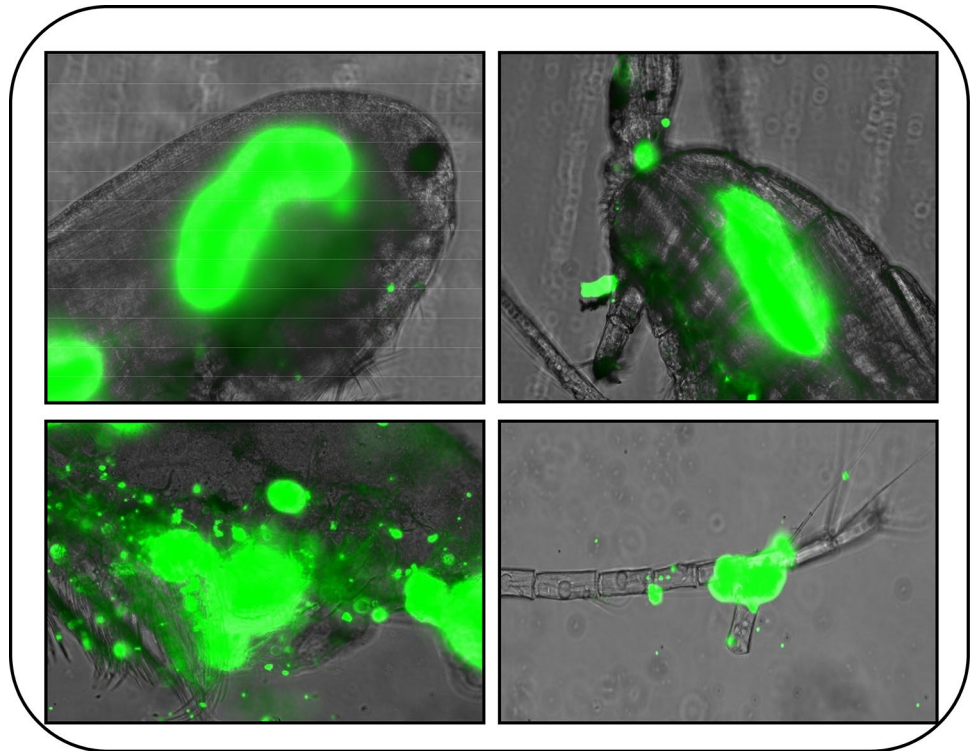


Plate 2 Microplastics accumulation in marine copepod *P. amandalei*



logarithmic to stationary phases in this study. Diatom (*A. subtropica*) was exposed to 2.0 μm size of MPs beads and their effects on photosynthesis and growth were determined every 48 h once for the period of 10 days. All tests were performed under controlled conditions with a start density (4.02×10^4 cells/ml) allowing for exponential growth during the experiment. Among these four different treatments, the highest MPs concentration of 75 $\mu\text{l}/100$ ml showed the lowest microalgal photosynthesis, growth rate and cell density followed by 50 and 25 $\mu\text{l}/100$ ml microplastics exposure treatments at the mid stationary phase. The growth rate, cell density and chlorophyll concentration were found to be increased from day 2 to day 6 after it was declined. The minimum of cell density, algal photosynthesis and growth rate were observed with the MP concentration of 25 $\mu\text{l}/100$ ml. Besseling et al. (2014) demonstrated that PS particles of a similar size (0.07 μm) inhibited the growth of the microalga *Scenedesmus obliquus*. The adsorption of the MPs beads on algae could block light from reaching the photosynthesis, similar studies were earlier carried out by Yufeng Mao et al. (2018) and Marc long et al. (2015). Sjollem et al. (2016) suggested that the nanoscale, particles (0.1 μm) become more easily undergo aggregation (Gigault et al. 2018) and (Galloway et al. 2017), which would cause more interaction between microplastics and algae. These results confirmed that the effect on microalgal growth decreased with increasing beads concentration and size. During the present investigation period, *T. suecica* showed the highest homo

aggregation compared to *A. subtropica*. The role of MPs in the fate of aggregates may vary depending on the MPs size, density and composition (Cózar et al. 2014).

Microalgae generate a wide range of lipids with nutritional value. As a result, microalgae play an important role in the aquaculture food chain, primarily as live feeds for larval culture (Brown 1997; Martínez-Fernández et al. 2006). Lipid composition and productivity depend on growth conditions such as growth phase (Xu et al. 2008), medium composition (Valenzuela-Espinoza et al. 2002), irradiance rate (Thompson et al. 1993), and temperature conditions (Renaud et al. 2002). In the present investigation, the total lipid was analyzed in *T. suecica* and *A. subtropica* (with and without MPs). The maximum concentration of lipid was observed in *A. subtropica* without MPs (Control) which may be due to the nitrogen depletion (Valenzuela 2013). The MPs containing microalgae not showed any variation in total lipid concentration. Similar findings were earlier reported in mussels by Smolders and Degryse (2002). Furthermore, we hypothesized that the aggregated microalgae may alter the lipid profile. However, there was a substantial change in total lipid mass of microalgae at end of the exposure period, but the lipid profiles of the control were significantly altered.

Microplastics have been found to be present in many parts of the marine food web, especially in the water column with various impacts on the zooplankton (Cole et al. 2015). This study investigated the effects of microplastic beads of 2 μm that might have on the filtration ability in

copepod *P. annandalei*. The present results showed that the copepod can ingest microplastics (2.0 µm diameter) in the absence of natural food. Microplastics were unknowingly ingested via filter-feeding and later egested in faecal pellets, typically within a matter of hours. Microplastics were found to be trapped between the external appendages of copepods owing to its accumulation on the external surface. The microscopic images of copepod clearly showed that 2.0 µm polystyrene beads clustered within the alimentary canal and aggregated between the setae and joints of external appendages. The presence of 2.0 µm polystyrene beads reduced the algal ingestion rate, nauplii production and survival rates of copepod *P. annandalei*. Similarly, Cole et al. (2013) reported *Temora longicornis* and *Centropages typicus* ingested microplastics with different sizes 7.3, 20.6 and 30.6 µm beads and *Acartia clausi*, *Calanus helgolandicus* ingested 7.3 µm beads affected the feeding and survival rates of the animals. The present study demonstrated that the minimum survival and nauplii production rate was found in microplastic ingested copepod. Prolonged exposure to the microplastics resulted in less egg production with reduced hatching success and survival rate was also reduced within 4–5 days in *P. annandalei*. The present findings shows that microplastics can impede copepod feeding and reduce the nauplii production rate and survival. These effects were most noticeable at 3–4 days after the ingestion of microplastics into the organisms. Lee et al. (2013) found that when exposed to 0.5 and 6 µm microplastics, the number of nauplii which hatched from eggs produced by the benthic copepod *Tigriopus japonicus* was reduced. Similar results have been observed by Bonnet et al. (2005), Cole et al. (2013, 2015). In adult *P. annandalei*, approximately 40% of energy derived from their food goes towards the buildup of their lipid store. (Marshall and Orr, 1955). The present study results concluded that the maximum concentration of lipid was shown in algae fed copepod (19%) and lipid contents were observed in the MPs exposure copepod (18%). Furthermore, there were no differences found in the total lipid concentration of copepods at end of the exposure period.

Conclusion

In the present study, two different microplastic beads were induced in a two-microalga, viz., *T. suecica* and *A. subtropica* over the 10 days experimental period. The significant PS beads aggregation was observed in *T. suecica* (than in the *A. subtropica*). The findings on the effect of MP beads absorption in total lipid production of *T. suecica* and *A. subtropica* showed not much variations observed in lipid production of control and MPs ingested microalgae. The microplastic ingested *P. annandalei*, showed less survival and nauplii production further, microplastic adsorption and consumed

copepods showed minimal changes in the lipid production. Higher trophic species that rely on the high lipid content of microalgae and copepods for their own sustenance may be affected by energetic deficits and decreased lifespan of microplastic exposed marine microalgae and copepods. The results concluded that better understand the density of bio-available microplastics in biota-rich waters, as well as to test whether environmentally relevant concentrations of plastic litter can impact keystone species such as *T. suecica*, *A. subtropica* and *P. annandalei*, as well as the consequences for commercially important predators.

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Declarations

Conflict of interest The authors declare that there is no conflict of interest.

Ethical approval This article does not contain any studies with animals performed by any of the authors.

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Biodegradation of low-density polyethylene and polypropylene by microbes isolated from Vaigai River, Madurai, India

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Abstract

The present study aimed to evaluate the microplastic degradation efficiency of bacterial isolates collected from Vaigai River, Madurai, India. The isolates were processed with proper methods and incorporated in to the UV-treated polyethylene (PE) and polypropylene (PP) degradation. Based on preliminary screening, four bacterial isolates such as *Bacillus* sp. (BS-1), *Bacillus cereus* (BC), *Bacillus* sp. (BS-2), and *Bacillus paramycoides* (BP) were proceed to further degradation experiment for 21 days. The microplastics were filled with bacterial isolates which is use microplastic (PE, PP) as carbon source for their growth and proceed for shake flask experiment were carried out by two approaches with control. The microplastic degradation was confirmed through their weight loss, increasing fragmentations and changes of surface area against control experiments (microplastic without isolates) also confirms degrading efficiency of isolated bacterial strains through non-changes in their weight and surface area. The highest degradation of PP and PE were observed in BP ($78.99 \pm 0.005\%$), and BC ($63.08 \pm 0.009\%$) in single approach, while in combined approach BC & BP recorded the highest degradation in both PP ($78.62 \pm 2.16\%$), and PE ($72.50 \pm 20.53\%$). The formation of new functional groups is confirming the biofilm formation in the surface area of microplastics by isolates and proving their efficiency in degrade the microplastics. The degradation of microplastic experiments should be cost effective and zero waste which is helpful to save the environment and the present findings could reveal the way to degrade the microplastics and prevent the microplastic pollution in aquatic environment.

Keywords Polymers · Microplastics · Bacterial isolates · Degradation · Polyethylene · Polypropylene

Introduction

The intrinsic characteristics like low production cost, and lifetime given outstanding milestone has reach 60 million tons plastic production per year (Plastics in Europe 2017). Among them 60% plastics was engaged with packaging industry, and construction sector while nearly 30% were ended as landfills. The anthropogenic activities in marine and terrestrial environment has cause severe plastic pollution

and their affects still increasing (Li et al. 2016; Ng et al. 2018). The plastic fragments were dispersed in marine environment with respect of depth, place and season and their concentration was varied habitat wise including water, sediments and even animals (Browne et al. 2011). Their diversity and density make negative impacts in the marine biota from lower order to top order which will affects the single animal to entire organisms (Sharma and Chatterjee 2017). The activities of plastic degradation also consider as major source of microplastic which was getting fragment as small particles and distributed in to the ocean and their rate of lose quite increasing than large size plastics (Alomar et al. 2016). There are many studies reported that, microplastics are consumed by wide range of marine organisms like phytoplankton, zooplankton, fish, crustaceans and they getting damages such as reproductive systems, liver inflammation, reducing growth rate and enzyme production (Batel et al. 2016; Lönnstedt and Eklöv, 2016; Lu et al. 2016; Fossi et al. 2016). And also the microplastics can have the ability to absorb the metals and other organic pollutants in their

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body from associated environment and it will act as bridge for transporting metals to consuming one (Brennecke et al. 2016). Hence, there is urgent need to degrade the microplastic from aquatic and other available environments, and to till date there are many methods has been tried to clean the microplastics with the help of chemical, biological methods. Among them, the degradation by microbes produce positive output in the range to reduce the concentration of plastics using polyethylene terephthalate as sole energy for their growth (Yoshida et al. 2016). Bhardwaj et al. (2012) stated that, microbial degradation for plastic being first choice for the researchers due to nil negative effects to the environment and also screening promising microbes to degrade plastics for safe and enhanced bioremediation. Freshwater microbes consisting wide range of microbes with huge capability to degrade pollutants which was proved early by many researchers (Bhardwaj et al., 2012; Yoshida et al. 2016). Hence, the present study, aimed to assess the efficacy of microplastic dissolution using microbial isolates from Vaigai River, India.

Materials and methods

Plastic materials

The degradation experiment were conducted by indoor condition and the plastic materials were obtained from the shop which is producing plastics for commercial purposes and the plastics were gruff by scissors. Then the broken plastics were filtered through sieve (250 nm, mesh no. 60) for segregate the large size plastics and the filtered plastic materials which is made of polypropylene, and polyethylene were identified. The UV (KEMI, KLFISS-2011) treatment has been implemented for 25 days before incorporated in to the experiment. The size of plastic materials were assessed with the help of inverted microscope (ACCU-SCOPE, EXI-310, TENSION, USA).

Collection and characterization of sediment samples

The sediment samples were collected from Vaigai River, Madurai, India with intervals of 1 cm and 0–6 cm depth with help of quadrant from the undisturbed area in river. The

collected sediment samples were stored into sterile plastic container and shifted to the laboratory for further experiment. The initial water quality parameters were estimated and given in the Table 1. All the experiments were carried out in triplicate.

Bacterial isolation and identification

To isolate the single colony bacterial species, the 9 mL of tap water mixed with 1 g of sediment sample. Then the mixture was incorporated in to the vortex mixture at 180 rpm for 3 h with the help of orbital shaker. Then the mixed suspension subjected to serial dilution followed by agar plating using nutrient agar followed by incubation for 24 h at 35–38 °C. Further, subculture were performed with single colonies using freshly prepared nutrient agar for isolating individual pure cultures. The triplicates experiments have been followed through the experiment further, biology GEN III microplate protocol were followed to identification of bacteria (Emerson et al. 2008).

Genomic DNA isolation, phylogenetic and sequence analyses

Genomic DNA extraction and isolation from bacterial isolates were carried out as per the protocol described by Rajasabapathy et al. (2018) with slight modification from Luna et al. (2006). The PCR product were amplified using eubacterial primer sets 1492R (5'-GGTTACCTTGTTACGACTT-3') and 27F (5'-AGAGTTTGTATCCTGGCTCAG-3') (Lane 1991). The amplified product was undergone for sequencing followed by deposition to GenBank through BankIt submission tool. The neighbour-joining method were used to construct the phylogenetic tree with the help of MEGA 6 software.

Experimental design

The microplastic degradation screening with bacterial isolates was carried out as per the method of Kannahi and Sudha (2013) with two types (individual and combined). The first set of experiments consisting individual microbial isolates and second set consisting combined microbial isolates. The schematic representation of experimental design was

Table 1 Water quality parameters of Vaigai River

pH	Temperature	Electrical conductivity	Total diss. solids	Dissolved oxygen	Total alkalinity	Total hardness	Calcium hardness	Chloride
7.1 ± 0.2	25.45 ± 0.49 °C	1128 ± 160 µS/cm	1030 ± 63 mg/L	9.12 ± 0.65 mg/L	310 ± 19 mg/L	354 ± 24 mg/L	96 ± 8 mg/L	190 ± 12 mg/L

Values shown are averages of three triplicates ± standard deviation, and the standard deviations were calculated from three repetitions

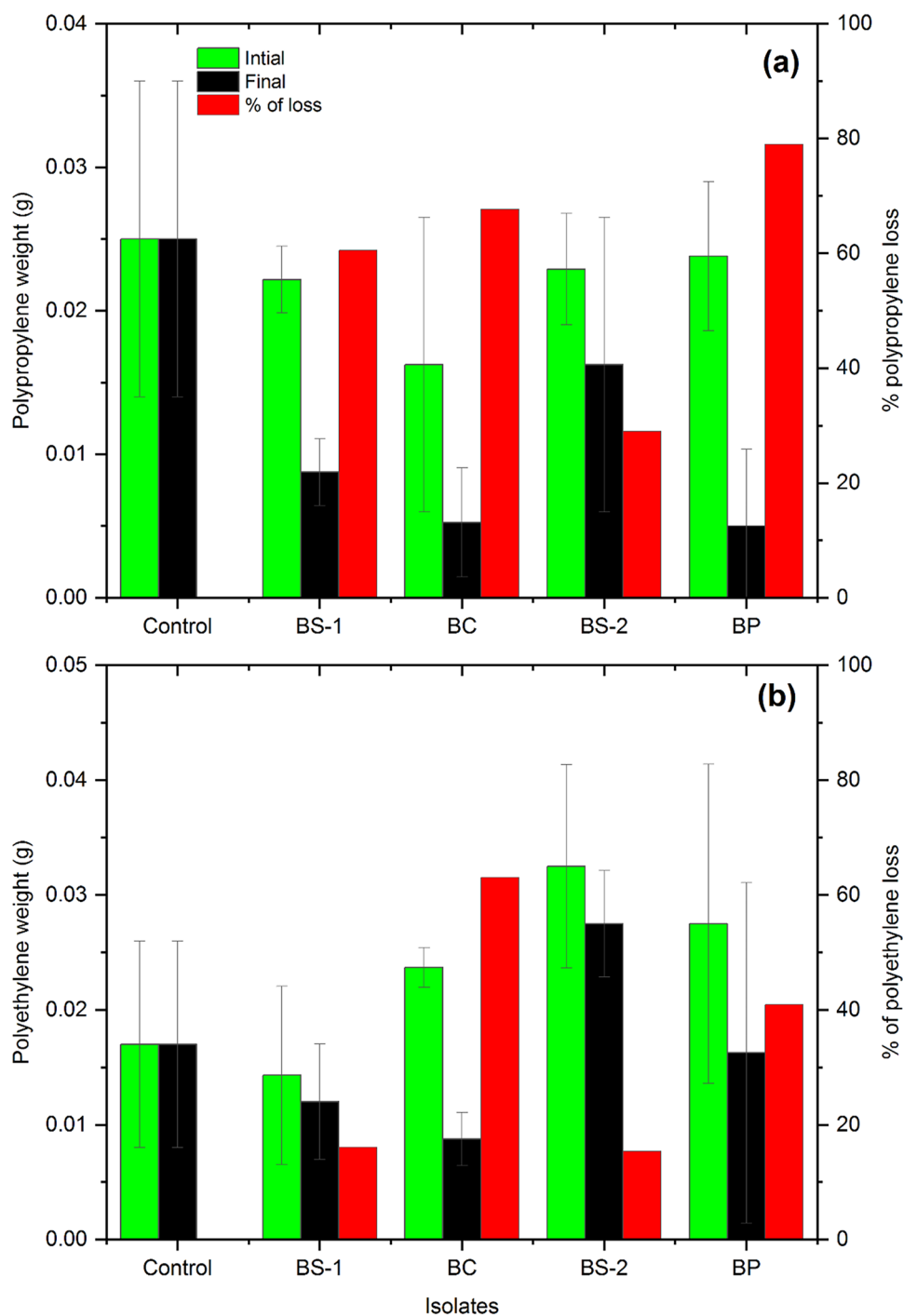
given in the Supp. Fig. 1. Briefly, the medium were applied with all essential nutrients to bacterial growth except carbon source. The PE and PP can act as carbon source for bacterial growth and four bacterial isolates were incorporated for the same. Then four specific isolates were inoculated as per the experimental design (given in the Supp. Figure 1) in to the nutrient agar media (NAM) with 0.5 g of plastic polymers followed by 21 days incubation in room temperature. The control setup also has been performed which is have only

plastic polymers without bacterial isolates and all the experiments were performed in triplicates.

Experimental approach

The microplastic degrading efficiency of bacterial isolates (individual and combine) was assessed as per the experimental design. In brief, the nutrient agar media were freshly prepared and stored in the 33 °C for 24 h and used to

Fig. 1 Biodegradation of PE and PP under four bacterial isolates (individual approach) for 21 days of culture period. Values shown are averages of three triplicates $\pm$ standard deviation, and the standard deviations were calculated from three repetitions. Control—without bacterial isolates, BS-1: *Bacillus* sp-1, BC: *Bacillus cereus*, BS-2: *Bacillus* sp-2, and BP: *Bacillus paramycoides*)



inoculate the bacterial isolates for obtaining pure cultures. Then inoculates were transferred to the conical flasks contain microplastic fragments and nutrients agar media. The experimental conical flasks were shifted to the rotary shaker (Rivotek, RC5100) and allowed to degradation at 28–30 °C for 180 rpm. The equal volume of isolates with same cell density were used to degrade the microplastic and their cell density were confirmed with measuring absorbance at 580 nm using UV-Spectrophotometer (Shimadzu 1900i). Then the isolates (4.0×10^8 CFU cells /mL) were transferred in to the 250 mL of round bottom conical flasks contains pre-weighed (0.5 g) microplastics and NGA. The control experimental flasks contain 0.5 g microplastics and NGA without isolates and all the experiments were conducted in triplicates. The total experimental duration was 21 days and pH, optical density of cultures, and microbial cell counts were estimated for every 7 days (0, 7, 14 and 21). The optical density measurement in experimental media is directly correlated with microbial growth.

General analysis

The dry weight of microplastics was estimated according to Auta et al. (2017a, 2017b). Briefly, the microplastics were harvested from the NGA broth through filtration using (1 mm) GFC filter paper followed by 70% ethanol rinsing. Then microplastics were dried using hot air oven at 50 °C for 24 h. The percentage of plastics degradation was calculated using following formula:

$$\text{Microplastic degradation (\%)} = \frac{\text{IWMP} - \text{FWMP}}{\text{IWMP}} \times 100$$

Here, IWMP denotes initial weight of microplastics, FWMP denotes final weight of microplastics. Further the collected data were proceed with first-order kinetic model for calculation of microplastic polymer reduction constant rate with the help of following formula:

$$K = -\frac{1}{D} \left(\ln \frac{\text{WRMP}}{\text{IWMP}} \right)$$

Here, K is the first order rate per day microplastic uptake, D denotes time in days, WRMP denotes the weight of remaining microplastic in experimental flasks (g), and IWMP denotes initial weight of microplastics. Then the generated data (K) were incorporated to the calculation of the half-life ($T_{1/2}$) as per the Alaribe and Agamuthu (2015).

$$T_{1/2} = \frac{\ln(2)}{K}$$

Beginning and end of the experiment, the FTIR Spectroscopy (Perkin Elmer, Spectrum RX I) analyses were carried in the microplastic polymers in the range of 400 to

4000 cm^{-1} frequency. The FTIR analysis was carried out in the selected microplastic polymers which contain bacterial isolates and without bacterial isolates (control). Intensity distribution analyses (particulate systems) were made to estimate the size of microplastic polymers after incubation with isolates. The conditions of measurement were provided in the Table 2. The scanning electronic microscopy (SEM) analyses were done to investigate the changes in surface morphology of microplastic polymers with influence of bacterial isolates during 21 days of experimental period. The control with sputter-coated gold layer and bacterial isolates microplastic biopolymers were assessed with the help of SEM (Carel Zeiss, EVO-18). The microplastic distribution was estimated by particle size analyzer (Micrometrics, Nano Plus).

Statistical analysis

The generated data were incorporated in to the analyses of variance (ANOVA) with the help of GraphPad Prism (version 8) and the generated data were expressed in the mean $\pm$ standard deviation.

Results and discussion

Isolation, identification and genomic evaluation of bacteria

From the river sediments, bacterial strains were isolated with proper method and their growth were confirmed with their efficiency to identification with individual isolates. Among the isolates, the four isolates from class Bacilli were proceed with pure stains. The isolated microbes are represent the river which is native bacteria from fresh water environment (Asmita et al. 2015; Mallika et al. 2017). The genomic DNA from four bacterial isolates was extracted and subjected to the agarose gel. Among the four isolates, two isolates procured genomic DNA with bp of 1065 and 823, respectively. Then the amplified sequences were analyzed using NCBI database and proceed with highest similarity of *Bacillus cereus* (MW881525) and *Bacillus paramycoides* (MW881520). The sequences were compared with same genus and other group of genus to find the differentiation. The obtained NCBI accession and identification were

Table 2 Measurement conditions for the analyses of intensity distribution

Temperature	Diluent name	Refractive index	Viscosity	Scattering intensity	Attenuator 1
25.0 °C	Water	1.3328	0.8878 cP	23,521 cps	0.42%

confirming that *Bacillus cereus*, *Bacillus paramycoides* (Supp. Figure 2) and other two isolates are named as *Bacillus* sp.-1, and *Bacillus* sp.-2.

Effect of bacterial isolates on polymer degradation

To degrade the polymer, four bacterial isolates (BS-1, BC, BS-2, and BP) were cultured in NAM with PE and PP which is consider as carbon source for bacterial growth. The multiplication of bacterial isolates could have the efficiency to degrade the polymers with considerable enzymatic activity and the loss of polymers weight explains that the, isolates can adopt their life in polymer associated environment. The biodegradable efficiency of bacterial isolates was assessed by two steps of experiment namely, separate and combined approach with bacterial isolates.

In vitro polymer degradation assay: individual and combined approach

Figure 1a, b shows that the degradation of PE and PP under four bacterial isolates which is utilized PE and PP as carbon source. After 21 days of incubation, the bacterial isolates are significantly reduce the weight of microplastics and their calculated rate of reduction constant (K) and half-life. Among the four isolates tested, the highest rate of weight lose were observed in *B. paramycoides* for polypropylene and *B. cereus* for polyethylene and the range of weight reduction were $78.99 \pm 0.005\%$ and $67.69 \pm 0.005\%$, respectively. Likewise, *B. paramycoides* and *B. cereus* resulted highest daily reduction rate such as 0.038 day^{-1} and 0.030 day^{-1} to reduce weight of polypropylene and polyethylene, respectively. The half-life by four bacterial isolates to reduce the polypropylene were 1.54 (BS-1), 1.01 (BC), 3.31 (BS-2), and 1.27 days (BP). In polyethylene degradation, the isolates such as BS-1, BC, BS-2, and BP have depicted 3.73, 1.58, 8.87, and 2.82 days, respectively (Table 3). Hence, the combined approach with four bacterial isolates, six combination were formulated and tested with addition of control (without isolate). Among the seven experimental combinations, the highest rate of microplastic degradation were observed in the combination of BC and BP for polypropylene, polyethylene and the percentage of degradation were $78.62 \pm 2.16\%$ and $72.50 \pm 20.53\%$ (Fig. 2a, b). The second best was observed in BS-1 and BP for polypropylene degradation ($51.50 \pm 16.98\%$) and BS-1 and BC for polyethylene degradation ($49.31 \pm 10.60\%$). Table 4 shows the removal constant rate for polypropylene, polyethylene which are highest values were observed in BC and BP and values were 0.037 day^{-1} and 0.033 day^{-1} respectively. Likewise, the lowest half-life was observed in BC and BP for polypropylene (2.07 days) and BS-1 and BC for polyethylene (1.73 days).

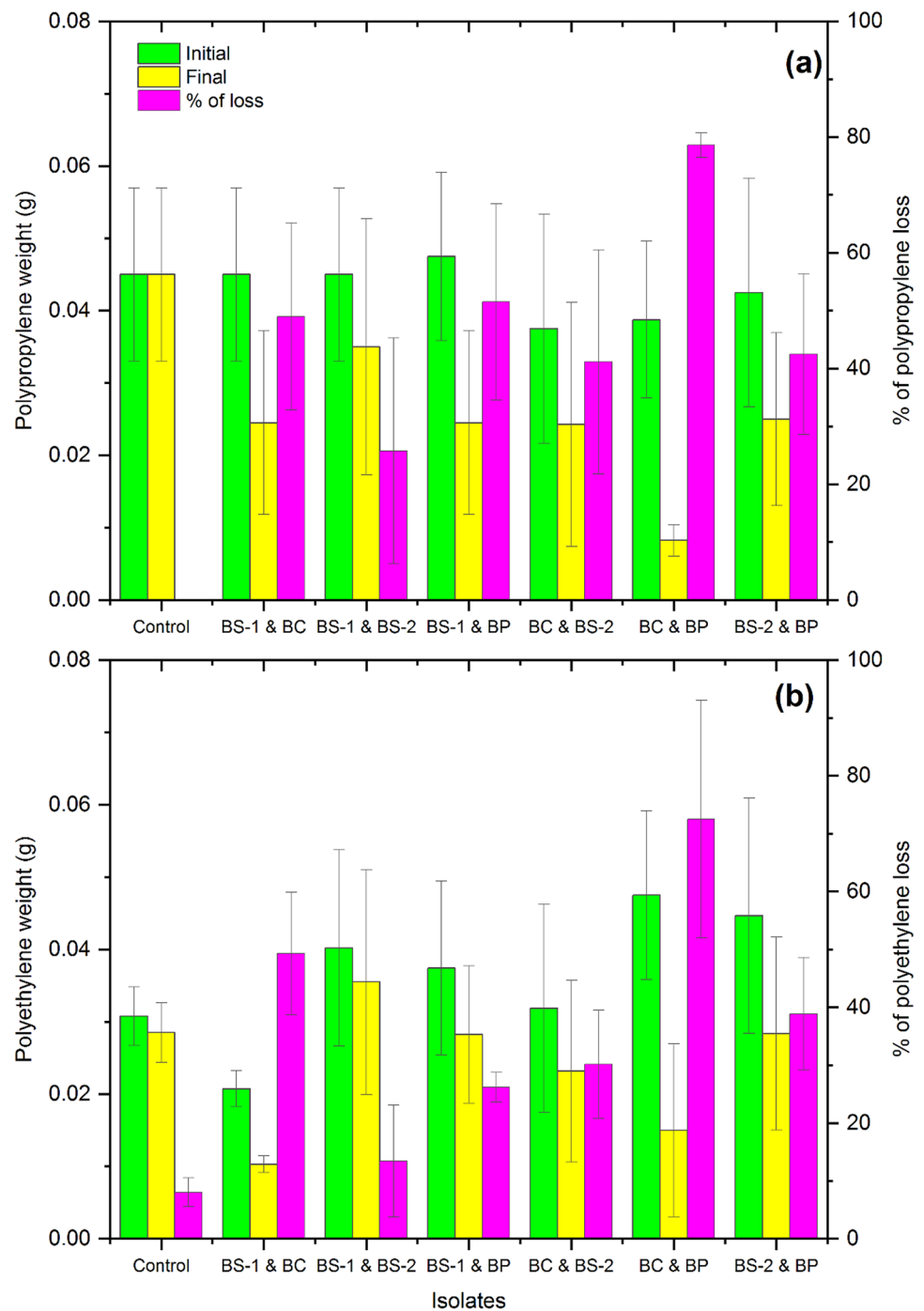
Table 3 Weight loss of polymer (PE & PP) and growth kinetics of four bacterial isolates by individual approach in microplastic-pervade nutrient agar media

Polymer type	Weight loss (%)	Removal constant (k) day^{-1}	Half-life (In $2/k$) (days)
<i>Polypropylene</i>			
Control	0	0	0
BS-1	60.54 ± 0.009	0.029	1.54
BC	67.69 ± 0.005	0.032	1.01
BS-2	29.04 ± 0.016	0.014	3.31
BP	78.99 ± 0.005	0.038	1.27
<i>Polyethylene</i>			
Control	0	0	0
BS-1	16.08 ± 0.012	0.008	3.73
BC	63.08 ± 0.009	0.030	1.58
BS-2	15.38 ± 0.028	0.007	8.87
BP	40.91 ± 0.016	0.019	2.82

Woodall et al. (2014) opined that the PP and PE are most common microplastics available in water environment include marine and fresh water environment and these plastics mostly available in the surface of the water environment due to their gravity level which contain 0.84 (PP) and 0.94 (PE). Likewise, Andrady (2011) stated that the PVC material mostly available in the bottom of the water environment which is contain higher gravity (1.025) than the PP and PE. Zettler et al. (2013) stated that selection of appropriate microbes for degradation of microplastic is important factor through the observation of higher richness in water column than the plastic associated one. In this case, the present study dealt with four different isolates to degrade the two (PP, PE) different plastics through shake flasks experiment. The UV treatment of microplastics could lead to loss of weight while associating with isolates which is increasing terminal double bond and carbonyl indices in plastic for easy exposure (Auta et al. 2017a,b). And also their attaching efficiency may vary based on their functional groups which is available in isolates (Harshvardhan and Jha, 2013). The experiment lasted for 21 days and the plastics were settled in the bottom of the flask from 6th day of the experiment due to their weight increasing by bio-fouling formation in the surface of the microplastics and the similar trend has been observed by Auta et al. (2017a, b).

B. paramycoides and *B. cereus* recorded highest weight loss while individual as well as combined approach and range between 72–79% for PP and 67–79% for PE and this weight reduction was comparably higher than the findings of Harshvardhan and Jha (2013) who made attempt on *K. palustris*, *B. pumilus*, and *B. subtilis* for PE reduction. The 21 days of incubation may increase bacterial colonization on the plastic surface and their production rate were confirmed

Fig. 2 Biodegradation of PE and PP under four bacterial isolates (combined approach) for 21 days of culture period. Values shown are averages of three triplicates $\pm$ standard deviation, and the standard deviations were calculated from three repetitions. Control- without bacterial isolates, BS-1: *Bacillus* sp-1, BC: *Bacillus cereus*, BS-2: *Bacillus* sp-2, and BP: *Bacillus paramycoides*)



by changing color of microplastics as agreed earlier by Hemjinda et al. (2007). Okpokwasili and Nweke (2005) stated that the increasing bacterial concentration, and modifying the combination of bacterial isolates could significantly inducing the polymer degradation.

The degradation of PE by bacterial isolates may extent up to certain limits and it depends on their binding efficiency in to the plastics (Helen et al. 2017a). While individual approach, the highest polypropylene degradation

was observed in the *B. paramycoides* than the *B. cereus* and this results were contrast with Helen et al. (2017a, b) who made attempt on polypropylene degradation by *B. cereus* and *S. globispora* and obtained highest degradation by *B. cereus* than the *S. globispora*. But, Sowmya et al. (2014a, b) has recorded highest polyethylene degradation by *B. cereus*. Helen et al. (2017a, b) opined that the weight loss of PP and PE during the incubation period was fully depends on the biological process occurring

Table 4 Weight loss of polymer (PE & PP) and growth kinetics of four bacterial isolates by combined approach in microplastic-pervade nutrient agar media

Polymer type	Weight loss (%)	Removal constant (k) day ⁻¹	Half-life (In 2/k) (days)
<i>Polypropylene</i>			
Control	0	0.000	0.00
BS-1 & BC	49.00 ± 16.16	0.022	4.15
BS-1 & BS-2	25.75 ± 19.54	0.011	8.50
BS-1 & BP	51.50 ± 16.98	0.023	4.12
BC & BS-2	41.17 ± 19.37	0.017	4.46
BC & BP	78.62 ± 2.16	0.037	2.07
BS-2 & BP	42.50 ± 13.89	0.020	4.34
<i>Polyethylene</i>			
Control	8.06 ± 2.50	0.004	17.48
BS-1 & BC	49.31 ± 10.60	0.024	1.73
BS-1 & BS-2	13.42 ± 9.66	0.006	14.44
BS-1 & BP	26.19 ± 2.55	0.012	6.41
BC & BS-2	30.15 ± 9.32	0.013	4.90
BC & BP	72.50 ± 20.53	0.033	2.92
BS-2 & BP	38.85 ± 9.73	0.089	5.145

in the media by isolates and not because of associate chemicals present in the media and the present results has been confirming the same. Among the two polymers, PP (78.99 ± 0.005%) was degraded successfully than the PE (67.69 ± 0.005%) by bacterial isolates in the present study and this results were clearly demonstrate that the binding and colony formation in surface of the polymers were quite successful in PP than PE by the isolates.

The combined approach of isolates for the degradation of polymers could revealed significant outcome in terms of loss of plastics weight and highest degradation were observed by BC and BP combination for both PP (78.62 ± 2.16%) and PE (72.50 ± 20.53%) degradation than other combinations (BS-1 & BC, BS-1 & BS-2, BS-1 & BP, BC & BS-2, and BS-2 & BP) tested. The combined approach has given successful results for polyethylene degradation which were higher than the individual approach by four isolates and the highest rate of reduction was only 67.69 ± 0.005% (BC) while combined approach were given nearly 78.62 ± 2.16% (BC & BP). On the other hand, polypropylene degradation was high in individual approach (78.99 ± 0.005% by BP) than the combined approach (72.50 ± 20.53% by BC & BP combination). Hence from the present study results we came to understand that the individual approach is better way to degrade the polypropylene especially with *B. cereus* while *B. cereus* combined with *B. paramycoides* could potentially degrade the polyethylene.

Growth profile of isolates on degradation chamber

Figure 3a–d shows the growth profile of four bacterial isolates (individual and combined approach) during the plastic degradation with 21 days of incubation period. In individual approach, the highest growth were observed in *B. cereus* isolates in the polypropylene (0.42 ± 0.005 abs) and polyethylene (0.42 ± 0.008 abs) contains chamber. Likewise, BC and BP combination revealed highest cells growth in polypropylene (0.67 ± 0.001 abs) and polyethylene (0.71 ± 0.003 abs) contains chamber. Among the two polymers, polyethylene provided higher cell growth than the polypropylene and its might be due to the availability of glucose content in their surface area. Similarly, the highest polyethylene degradation observed in BC and BP which is given highest cell growth rate than other combinations. The growth curve of *B. cereus* were gradually increased up to 18th day of experiment similar to other strains in both polyethylene and polypropylene chambers. But there is an elevated growth were observed on 21st day in polypropylene and 18th day in polyethylene. In combined approach, the cell growth of BC and BP combination was higher than the first day experiment and the same trend has been observed until end of the experiment (21 days). Auta et al. (2017a, b) stated that the growth response of isolates may depends on the amount of surface area available in polymers and their rate of degradations. Similar findings were observed in this present study while the highest rate of polymer reduction were observed in BC in PE degradation (single approach), BC and BP in PE and PP degradation (combined approach). Hence, in single approach, highest growth was observed in *B. cereus* while highest degradation were recorded by *B. paramycoides*.

Fourier transform infrared (FTIR) analysis of microplastic polymers

The degradation of polymers by biotic source may start with carbonyl groups formation by oxidation process. The consumption of citric acids for formation of CO₂ and H₂O which will produce the carboxylic groups with the help of b-oxidation and the citric acids was catalyzed by biotic sources like microorganisms (Albertsson et al. 1987; Esmaeili et al. 2013). Esmaeili et al. (2013) opined that the degradation of polymers may depends and confirmed by monitoring the formation and disappearance of double bonds and carbonyl groups using FTIR analyses. The FTIR analyses (Perkin-Elmer Spectrum, RX I, United Kingdom) were carried out in the polymers with isolates and without isolates with standard frequency limits i.e.: 4000 to 400 cm⁻¹. The FTIR analyses were carried out to find the modifications in chemical properties of polymers with influence of bacterial isolates which is confirming the degradation process. Based on the degradation of polymers by isolates, the four experimental

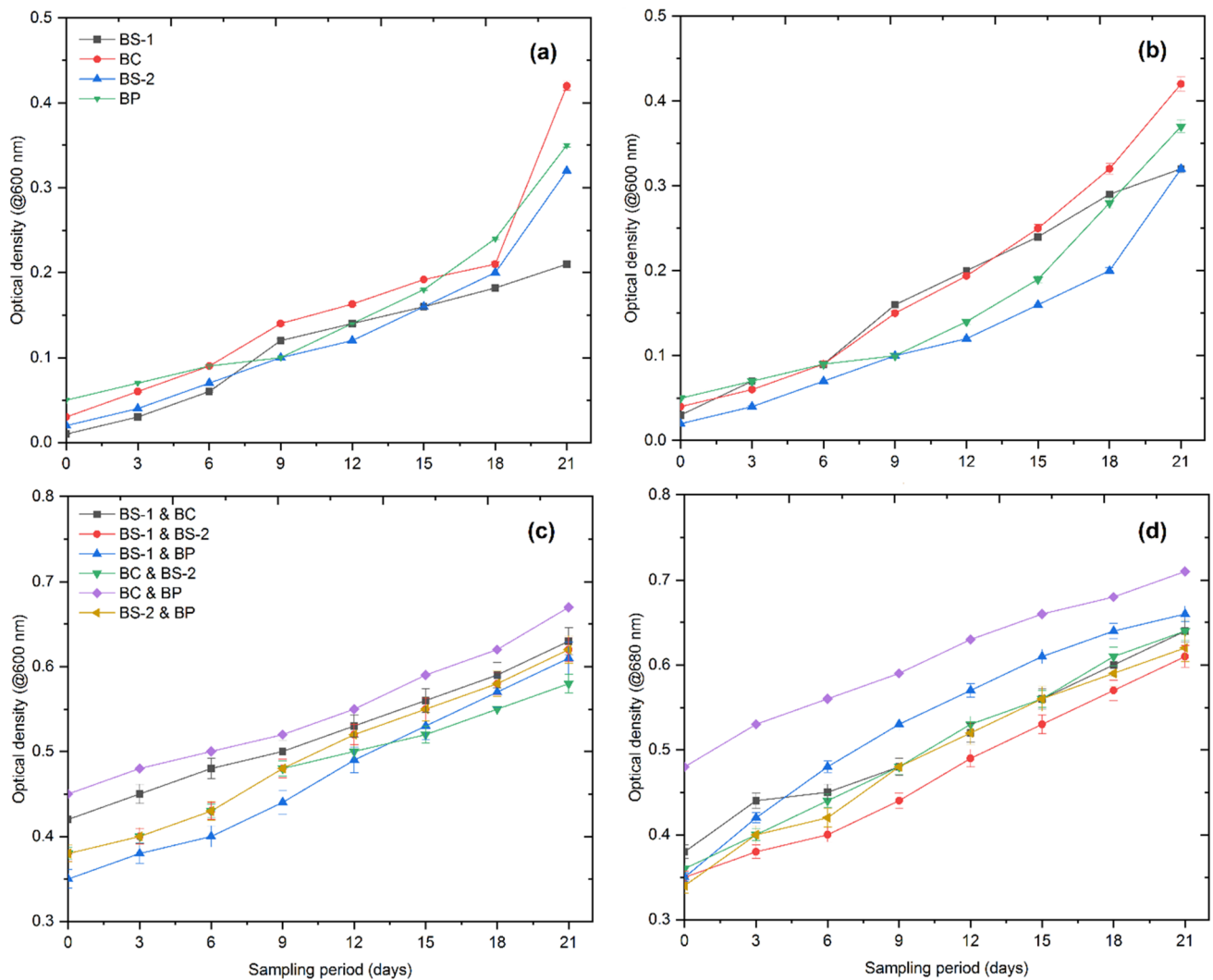


Fig. 3 Growth curve of four bacterial isolates during degradation studies for 21 days of culture period. **a** polypropylene; **b** polyethylene for individual approach; **c** polypropylene; **d** polyethylene for combined approach. Values shown are averages of three triplicates $\pm$ standard deviation, and the standard deviations were calculated from three repetitions. BS-1: *Bacillus* sp-1, BC: *Bacillus cereus*, BS-2: *Bacillus* sp-2, and BP: *Bacillus paramycoides*)

polymers such as BC, BP and BC & BP from polypropylene degradation chambers were proceed to FTIR analyses including control. Figure 4 shows the FTIR spectra of four different polypropylenes after 21 days of incubation which is named as control (without bacterial isolates), BC (*B. cereus*), BP (*B. paramycoides*), and BC & BP (*B. cereus* and *B. paramycoides*). By isolates, 14, 12, 12, and 20 peaks were found recorded in polypropylene by treating control, BC, BP, and BC & BP, respectively. Among the four, the polymers treated with *B. cereus* and *B. paramycoides* by combined approach recorded highest peak with differed from the other experiments. From the start, BP and BC recorded peaks from 617.24 and 618.06 cm^{-1} respectively which is unnamed groups. Further, benzene derivative compounds were recorded in control, BC, and BC & BP polymers was

observed at 718.34 cm^{-1} , 717.92 cm^{-1} , and 719.01 cm^{-1} respectively. Then the strong C-H bending group were observed in control (779.63 cm^{-1}), BC (779.77 cm^{-1}), and BP (779.73 cm^{-1}) treated polymers. In all four polymers, secondary alcohol compounds were identified which is comes under C–O stretching groups and the wavelength between from 1115.87 cm^{-1} and 1117.04 cm^{-1} . BC- and BP-treated polymers were found recorded alkyl aryl ether compounds (C–O stretching group), and phenol compounds from O–H bending group. In all four tested polymers, sulfonamide compounds from S=O stretching group were recorded and the wavelength ranged between 1352.61 and 1366.63 cm^{-1} .

The unique peaks from BP, and BC & BP treated polymers at 1463.32 cm^{-1} , and 1464.71 cm^{-1} found recorded and this peak shows that the essential ingredients of *B.*

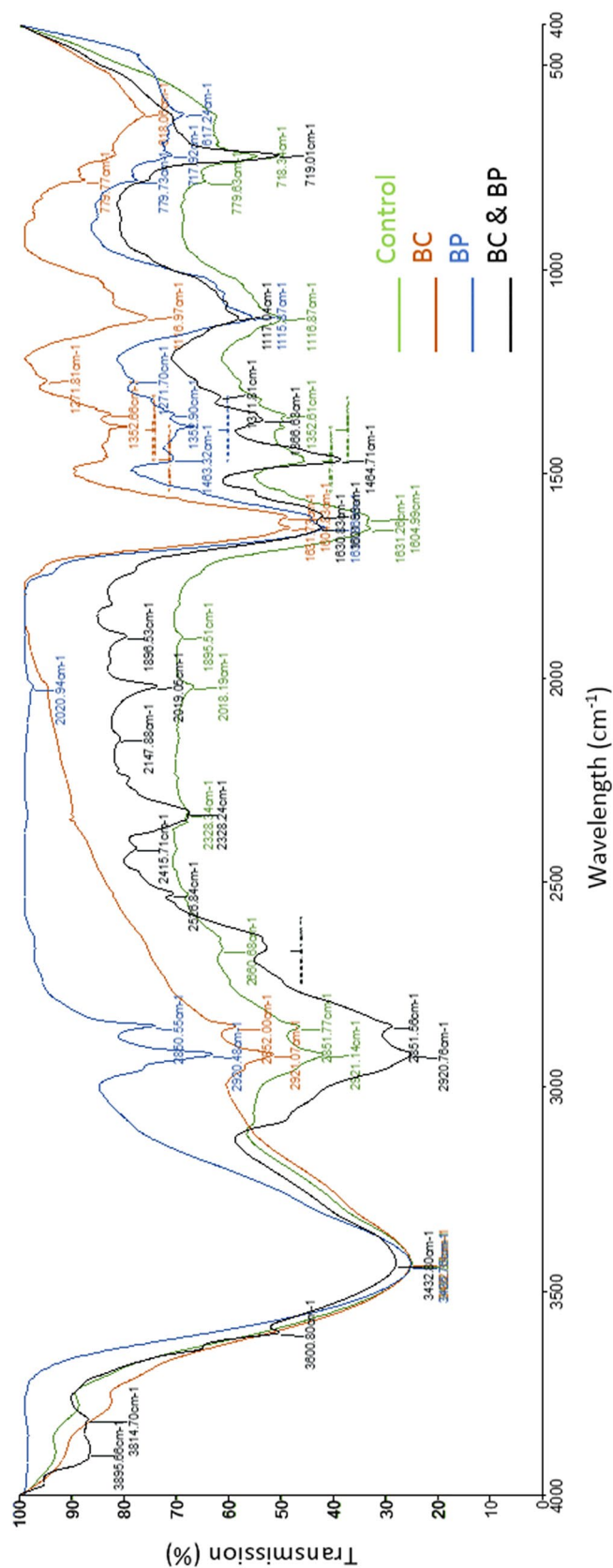


Fig. 4 FT-IR spectra of control and bacterial isolates treated polypropylenes under 21 days of incubation period. Control-polypropylene without bacterial isolates after 21 days of incubation; BC-polypropylene treated with *B. cerus* in single approach after 21 days of incubation; BP-polypropylene treated with *B. paramycooides* in single approach after 21 days of incubation; BC & BP-polypropylene treated with *B. cerus* and *B. paramycooides* in combined approach after 21 days of incubation

cereus and *B. paramyoides*. This peak also shows that the chemical composition of protein, amino acids, and neutral polysaccharides from bacterial isolates (Ma et al. 2014; Auta et al. 2017a, b). The conjugated alkene compounds from C=C stretching group were found recorded from all isolates tested polymers includes control which is wavelengths from 1600 to 1650 cm^{-1} . The weak aromatic compounds from C-H bending group were recorded in control (1895.51 cm^{-1}) and BC & BP (1896.53 cm^{-1}) treated polymers. Except BC-treated polymers, in other polymers (control, BP, and BC & BP) the strong isothiocyanate compounds from N=C=S stretching group were recorded from the wavelengths of 2018.19 cm^{-1} , 2019.05 cm^{-1} , and 2020.94 cm^{-1} . The azide compounds (2147.88 cm^{-1}) from N=N=N stretching group was found only in BC- & BP-treated polymers, and un-identified compounds were recorded from the peaks of 2415.71 cm^{-1} , and 2526.84 cm^{-1} . The strong carbon dioxide compounds from O=C=O stretching groups were recorded in control and BC & BP treated polymers. There is medium alkane compounds from C-H stretching group from the wavelengths from 2850.55 to 2921.14 cm^{-1} found recorded in all treated polymers. In control, BC, and BC & BP treated polymers were found recorded the strong alcohol compounds from O-H stretching groups (3432.70 cm^{-1}).

In BC & BP treated polymers, medium and sharp alcohol compounds from O-H stretching groups were identified in the wavelengths of 3600.80 cm^{-1} , 3814.70 cm^{-1} , and 3895.66 cm^{-1} . In this study, BC & BP treated polymers reported more number of carbonyl, alcohol, phenol, and hydroxyl groups which is increasing hydrophilicity of bacterial isolates and could enhance the degradation of plastics (Wilkes and Aristilde, 2017). Among the 4 different polymers tested, BC & BP treated polymers reported 6 new peaks than the control (14 peaks), BC (12 peaks), and BP (peaks) treated polymers. Sekhar et al. (2016) stated that formation of new peaks or functional groups which is reflected the alteration of side chain activities due to the various activities by bacterial isolates. And the observation in new peaks from bacterial isolates-treated polymers could reveal the introduction of oxidation products through various functional groups by degradation activities. Shah et al. (2008) and Wilkes and Aristilde (2017) opined that the new functional groups in polymers which clear evidence of b-oxidation and tricarboxylic acid

cycle process which is enhancing the polymers degradation process.

Intensity distribution analyses

Supp. Figure 3 shows the results of intensity distribution analyses for the polypropylenes with and without isolates for 21 days of incubation. The lowest distribution diameter (nm) is considered as the best polymers degradation by isolates or any biota. Among the four selected polymers (control, BC, BP, and BC & BP) tested, the lowest average distribution (10,463.4 $\pm$ 14,823.9 nm) were observed in the CP inoculated polymers with residuals values of 6.829e–003. Hence, BC & BP, BC, and control occupied the following positions and the values were 11,421.6 $\pm$ 16,331.4 nm, 11,950.8 $\pm$ 17,307.8 nm, and 20,236.6 $\pm$ 24,120.2 nm, respectively. Table 5 shows the cumulant results of polymers treated by various combination of isolates. The average diameter, polydispersity index, diffusion const. of microplastics was low in the BP isolated treated one followed by BC & BP, BC, and control. From these analyses, we confirming the polypropylene degradation was high in the BP followed by BC & BP treated chamber. Supp. Figure 4. Shows the microscopic view of microplastics at end of the experiment (21st day) and these photographs confirms that the degradation of microplastics can be done by bacterial isolates. The large quantity of fragments were observed in the BC & BP consisting microplastics followed by BP consisted one. The control container confirms without isolates, the microplastics did not cause any degradation while other bacterial isolates contains chambers contains fragmented microplastics.

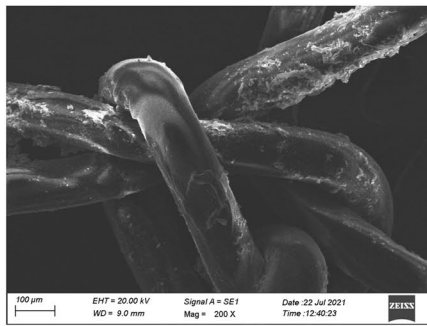
Scanning electronic microscopic analyses

The observation of microplastics through SEM analyses on bacterial isolates (BC, BP, BC & BP) and control (without isolates) were carried out after 21 days of incubation (Fig. 5). The SEM image microplastics contain bacterial isolates, have many numbers of cracks, grooves, holes, and their shape is getting much damage. Also, in bacterial contains microplastics chamber, did not find any full shape microplastics and this results provide solid evidence for erosion of microplastics due to the degrading efficiency of isolates. The same time control chamber (without isolates) did not cause any damage in their surface area. The biodegradation of microplastics using microbes conducted by early workers was given in Table 6.

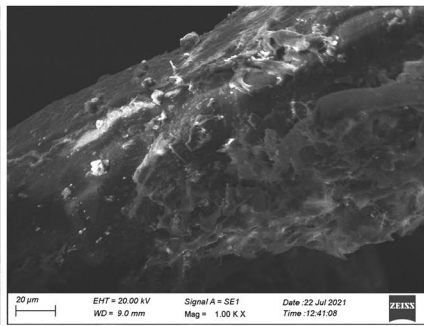
Table 5 Cumulants results of polypropylene during degradation with and without bacterial isolates for 21 days of incubations

Parameters	Control	BC	BP	BC & BP
Diameter (nm)	5620.3	5247.5	4497.7	4967.5
Polydispersity Index	1.520	1.469	1.390	1.419
Diffusion const. (cm^2/s)	8.752e–010	9.374e–010	1.094e–009	9.903e–010

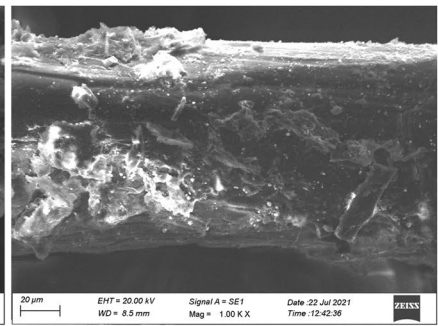
Control



100 μm Mag = 200 X

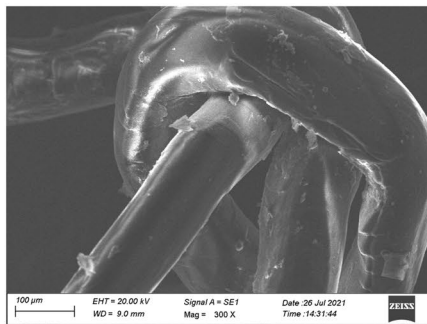


20 μm Mag = 1.00 KX

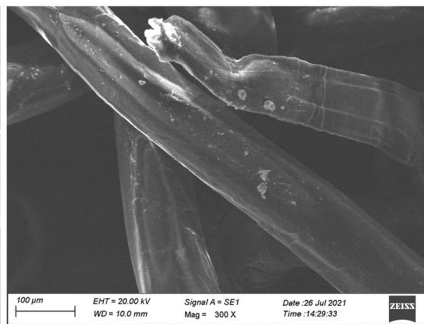


20 μm Mag = 1.00 KX

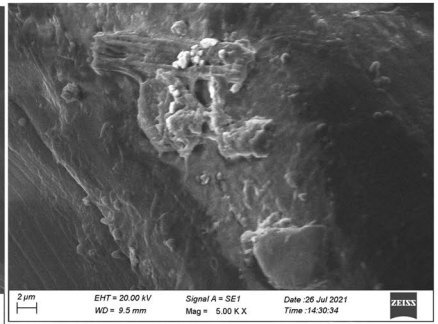
BC



100 μm Mag = 300 X

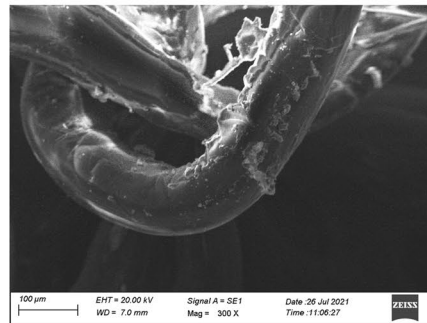


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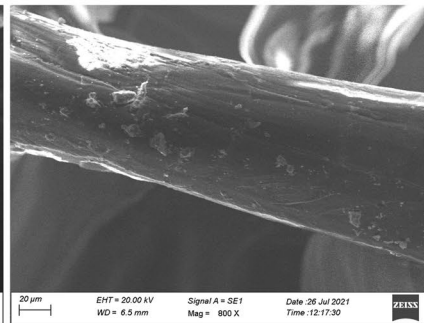


2 μm Mag = 5.00 KX

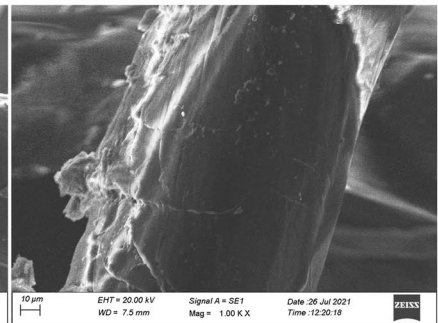
BP



100 μm Mag = 300 X

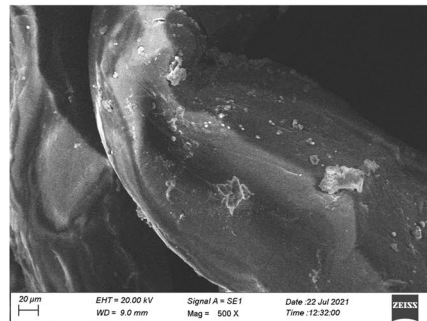


20 μm Mag = 800 X

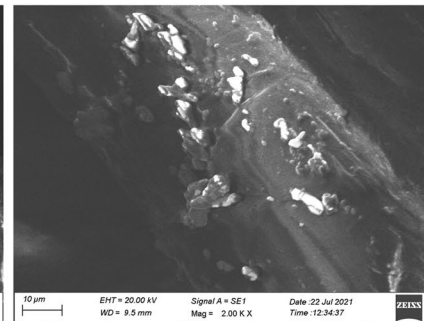


10 μm Mag = 1.00 KX

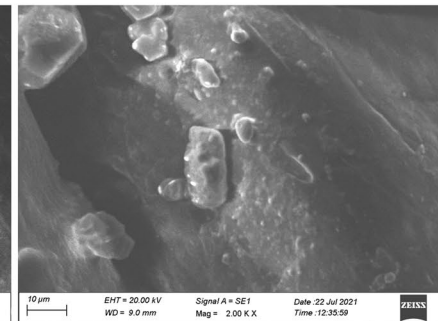
BC & BP



20 μm Mag = 500 X



10 μm Mag = 2.00 KX



10 μm Mag = 2.00 KX

Fig. 5 SEM images shows the degradation of polypropylene using bacterial strains and compared with control. Control—without bacterial culture; BC—*Bacillus cereus* degraded polypropylene; BP—

Bacillus paramycoides degraded polypropylene; BC & BP—mixed culture of *Bacillus cereus* & *Bacillus paramycoides* degraded polypropylene

Table 6 Biodegradation of microplastic by microbes conducted by early researchers

S. No.	Strain name	Type of plastic	Source	Nature of treatment	Weight loss (%)	References
1	<i>Bacillus Pseudonocardia</i>	PE	Marine	Indigenous Bioaugmented	7 11	Syranidou et al. (2019)
2	<i>Bacillus cereus</i> <i>Bacillus gottheilii</i>	PE, PET, PP, PS	Marine	Individually	1.6 (PE), 6.6 (PET), 7.4 (PS) 6.2 (PE), 3.0 (PET), 3.6 (PP), 5.8 (PS)	Auta et al., (2017a, b)
3	<i>Alcanivorax borkumensis</i>	LDPE	Marine	Individually	3.5	Delacuvellerie et al. (2019)
4	<i>Bacillus cereus</i> <i>Sporosarcina globispora</i>	PP	Marine	Individually	12 11	Helen et al. (2017a, b)
5	<i>Bacillus cereus</i> <i>Bacillus paramycoides</i>	PE, PP	Fresh	Individually Combined	63.08 (PE), 78.99 (PP) 78.62 (PP), 72.50 (PE)	Present study

PE polyethylene, PET polyethylene terephthalate, PP polypropylene, PS polystyrene, LDPE low-density polyethylene

Sowmya et al. (2014) already suggested the SEM analyses on the microplastic degradation by bacterial isolates is best tool to confirm the degradation with their pores, cavities and destructions. Similarly, Lucas et al. (2008) have observed the pores formation, cracks, color changes, surface area modifications in surface of the microplastics is considers as estimation of microplastic degradation.

Conclusion

The present study reveal the issue of microplastic accumulation in the aquatic environment. The four bacterial isolates were incorporated in to the polymers degradations (PE, PP) by single and combined approach with control which does not contains bacterial isolates. The growth patterns of four bacterial isolates in single and combined approach explains that the microplastics can be served as raw carbon source for the growth of bacterial isolates. The formation of new functional groups and disappearance of some existing functional groups explaining the biofilm formation and degrading activities on surface of the microplastics by bacterial isolates.

Microscopic and SEM image confirms the broken fragments, wholes, and cracks on the microplastic surface which could reveal the efficiency of bacterial isolates. Single and combined approach of bacterial isolates are efficiently reduce the microplastic weights by 21 days of incubation. Hence, the approach were used in this study could consider as potential method to degrade microplastic and this method might be a low cost and environmentally feasible. Further, optimization and depths on these related study could enhance the microplastic degradation in potential manner.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00203-021-02592-0>.

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Author contributions KND: conceptualization, methodology, resources, writing—original draft, writing—review and editing. PR: formal analysis. PS: conceptualization, methodology, writing—review and editing. SDK: formal analysis, writing—review and editing. NK: formal analysis. JR: writing—review and editing. PP: writing—review and editing.

Declarations

Conflict of interest The authors have declared that no conflict of interest found in this work.

Ethical approval This article does not contain any studies with animals performed by any of the authors.

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