

IMPACTS OF COMBINED TOXICITY OF MICROPLASTICS AND DIESEL POLLUTION IN MARINE BRINE SHRIMP (*Artemia salina*)

Submitted in partial fulfilment of the requirements for the award of

Bachelor of Science in Microbiology

by

FELICITA. S (40300076)

SUNMATHI. A. S (40300068)



**DEPARTMENT OF BIOTECHNOLOGY
SCHOOL OF BIO AND CHEMICAL ENGINEERING**

SATHYABAMA

INSTITUTE OF SCIENCE AND TECHNOLOGY

(DEEMED TO BE UNIVERSITY)

Accredited with Grade “A” by NAAC

JEPPIAAR NAGAR, RAJIV GANDHI SALAI,

CHENNAI -600119

May- 2023



SATHYABAMA

INSTITUTE OF SCIENCE AND TECHNOLOGY
(DEEMED TO BE UNIVERSITY)

Accredited with "A" grade by NAAC | 12B Status by UGC | Approved by AICTE
Jeppiaar Nagar, Rajiv Gandhi Salai, Chennai – 600 119
www.sathyabama.ac.in

DEPARTMENT OF BIOTECHNOLOGY

BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of **Felicita. S (40300076)**, **Sunmathi. A. S (40300068)** who carried out the project entitled IMPACTS OF COMBINED TOXICITY OF MICRO PLASTICS AND DIESEL POLLUTION IN MARINE BRINE SHRIMP (*Artemia salina*) under my / our supervision from

Internal Guide

Dr SUDHA. S
ASSISTANT PROFESSOR
DEPT. OF BIOTECHNOLOGY

SIGNATURE:

Head of the Department

Dr V. RAMESH KUMAR

External Guide

Dr M. RAVI
SCIENTIST-C
CENTRE FOR OCEAN
RESEARCH

SIGNATURE:

Submitted for Viva-voce Examination held on 9/5/2023

Internal Examiner

External Examiner

DECLARATION

Fellicita. S (40300076) hereby declare that the Project Report entitled IMPACTS OF COMBINED TOXICITY OF MICRO PLASTICS AND DIESEL POLLUTION IN MARINE BRINE SHRIMP (*Artemia salina*) done by me under the guidance of **Dr Sudha S** and **Dr M. Ravi** at Sathyabama Institute of Science and Technology is submitted in partial fulfilment of the requirements for the award of Bachelor of Science degree in Microbiology.

DATE: 29/4/2023

PLACE: CHENNAI



SIGNATURE OF THE CANDIDATE

ACKNOWLEDGEMENT

I am pleased to acknowledge my sincere thanks to the Board of Management of SATHYABAMA for their kind encouragement in doing this project and for completing it successfully. I am grateful to them.

I convey my thanks to **Dr V. Ramesh Kumar**, Head of the Department, Dept. of Biotechnology, School of Bio and Chemical Engineering for providing me with necessary support and details at the right time during the progressive reviews.

I would like to express my sincere and deep gratitude to my Project Guide **Dr. M. Ravi**, Assistant professor (Research), Centre for Ocean Research. I would also like to thank **Mr. S. Sakthinarendran Sai** for his valuable guidance, suggestions and constant encouragement which paved the way for the successful completion of my project work.

I wish to express my thanks to all Teaching and Non-teaching staff members of the Department of Biotechnology who were helpful in many ways for the completion of the project.

ABSTRACT

Over the last few decades, oceanic pollution has been a matter of significant threat to the environment. The ocean has been polluted by many factors including plastics, oil spills, pesticides and heavy metals. Plastics, which are dumped into the ocean, degrade into Macroplastics, Microplastics (MP) and Nanoplastics. Microplastics can easily be ingested by zooplankton, which resides on the surface of the ocean. During an oil spill, these zooplanktons ingest the particles present in crude oil. In both these cases, the particles which are ingested by the organisms cause a range of morphological and physiological characteristic changes. This study is aimed at analysing the combined toxicity of microplastics and diesel on zooplankton (*Artemia Salina*). The range finding test was performed for 24h and 48h using 5 different concentrations of MPs and Diesel separately to determine the lethal concentration (LC_{50}). The LC_{50} value for acute exposure was determined to be 10^5 particles/ml for microplastics and 10 μ l/ml concentration for diesel. Four different types of medium were used (Control, MP, Diesel, MP and Diesel combined) as the exposure media. Subsequent analysis will be performed to estimate the microflora present in the organism's gut following exposure to the medium. The outcomes of the aforementioned study will provide us with a comprehensive understanding of the detrimental effects of microplastics and diesel emitted into the oceans.

Keywords: Microplastic; Diesel; combined toxicity; *Artemia Salina*; Static acute toxicity

CHAPTER NUMBER	TITLE	PAGE NUMBER
	ABSTRACT	v
	List of abbreviations	ix
	List of figures	xi
	List of tables	xii
1	INTRODUCTION	1
	1.1- Plastics	2
	1.1.1 - History of Plastics	2
	1.1.2 - Effects of Plastic pollution	3
	1.1.3 - Different types of plastics	3
	1.2 - Microplastics	4
	1.2.1 - Impacts of microplastics in marine pollution	5
	1.3 - Oil Spill	6
	1.3.1 - Characteristics of Petroleum and Crude Oil	6
	1.3.2 - Major Oil Spills related to marine environment	7
	1.3.3 - Impacts of Oil Spills on marine biodiversity	9
	1.3.4 - Impacts of Oil Spill in the planktonic community	9
	1.3.5 - Oil toxicity	9
	1.4 - Brine Shrimp	10
	1.4.1 - Morphology	10

	1.4.2 - Taxonomy	10
	1.4.3 - Ecology	10
	1.4.4 - Reproduction	11
	1.4.5 - History of <i>Artemia</i>	12
	1.4.6 - <i>Artemia</i> genus in ecotoxicology	12
	1.5 - Microbial composition	13
2	REVIEW OF LITERATURE	14
3	AIM AND SCOPE OF THE PRESENT INVESTIGATION	20
	3.1 - Aim	20
	3.2 - Scope	20
4	MATERIALS AND METHODS	21
	4.1 - Toxicant materials	21
	4.2 - Hatching of the test species	21
	4.3 - Toxicology study	22
	4.3.1 - Toxicity media preparation	22
	4.4 - Range finding test	22
	4.5 - Probit analysis	22
	4.6 - Microbial study	22
	4.7 Colony morphology and colony counting	23
	4.8 Streak plate technique	23
	4.9 Gram staining	23
	4.10 Motility	24

	4.11 Acute gut exposure analysis	25
5	RESULTS AND DISCUSSION	26
	5.1 Range finding test	26
	5.2 Probit analysis	27
	5.3 Microbial study	29
	5.4 Colony morphology and colony counting	29
	5.4.1 Spread plate results	30
	5.4.2 Streak plate results	31
	5.5 Gram staining	32
	5.5.1 Negative control	33
	5.5.2 Diesel	33
	5.5.3 Microplastics	34
	5.5.4 Diesel + Microplastics	34
	5.6 Motility test	35
	5. Acute gut exposure analysis	35
	DISCUSSION	37
6	SUMMARY AND CONCLUSION	39
	REFERENCE	41

ABBREVIATIONS

UNESCO - United Nations Educational and Cultural Organization

GESAMP - Group of Experts on the Scientific Aspects of Marine Pollution

PVC - Polyvinyl chloride

PE- Polyethylene

PP - Polypropylene

PET - Polyethylene terephthalate

PS - Polystyrene

PA - Polyamide

MaP - Macroplastics

MP - Microplastics

NP - Nanoplastics

PPT - Parts per thousand

GSL - Great Salt Lake

CAT - Catalase

SOD - superoxide dismutase

ROS - Reactive oxygen species

B(a)P - Benzo(a)Pyrene

PAHs - Polycyclic aromatic hydrocarbons

3- mPhe - 3- Methyl phenanthrene

Ret - Reten

2-mAnt - 2-methyl anthracene

Phe - phenanthrene

Ant - Anthracene

FSW - Filtered Seawater

LC₅₀ - Lethal concentration 50

PBS - Phosphate buffered saline

NC - Negative control

D - Diesel

MP - Microplastic

μl - Microliter

RE - Regression equation

SE - Standard error

LIST OF FIGURES

FIGURE NUMBER	CONTENT	PAGE NUMBER
Figure 1.1	Global plastics production (1950 - 2019)	1
Figure 1.2	Micro-nanoplastics, Microplastics and nanoplastics increase in the ocean (2013 - 2021)	4
Figure 1.3	No.of oil spills that occurred globally by tankers (1970 - 2021)	6
Figure 1.5	Location of top 20 major oil spills	7
Figure 1.6	2017 Chennai oil spill location	8
Figure 1.7	Steps in the life cycle of <i>Artemia</i>	11
Figure 4.1	Hatching of cyst	21
Figure 4.2	Nutrient broth inoculated with 3hr fresh streak plate culture for a motility test	24
Figure 5.2	MP graph for range finding test	26
Figure 5.4	Diesel graph for range finding test	27
Figure 5.6	Probit graph results	28
Figure 5.10	Negative control (Spread plate results)	30
Figure 5.11	Diesel (Spread plate results)	30
Figure 5.12	Microplastics (Spread plate results)	31

Figure 5.13	Diesel + Microplastics (Spread plate results)	31
Figure 5.14	Negative control (Streak plate results)	31
Figure 5.15	Diesel (Streak plate results)	31
Figure 5.16	Microplastics (Streak plate results)	32
Figure 5.17	Diesel + Microplastics (Streak plate results)	32
Figure 5.19	Negative control (Gram staining results)	33
Figure 5.20	Diesel (Gram staining results)	33
Figure 5.21	Microplastics (Gram staining results)	34
Figure 5.22	Diesel + Microplastics (Gram staining results)	34
Figure 5.24	Acute gut exposure analysis results	36

LIST OF TABLES

TABLE NUMBER	CONTENT	PAGE NUMBER
Table 1.4	No.of oil spills over 7 tonnes from 1970 to 2013	7
Table 5.1	Microplastics LC50 value results	26
Table 5.3	Diesel LC50 value results	27
Table 5.5	Probit results	27
Table 5.6	Probit analysis results for Microplastics	28
Table 5.7	Probit analysis results for Diesel	29
Table 5.9	Colony morphology and colony count results	30
Table 5.18	Gram staining results	32
Table 5.23	Motility test results	35

CHAPTER - 1

INTRODUCTION

Different varieties of life that exist on Earth are referred to as biodiversity or biological diversity. The world's biodiversity may be found primarily in the ocean. The marine ecosystem is estimated to have about 2,210,000 species of both animals and plants. Even though it incorporates the majority of the global biodiversity, it is also the most completely obliterated, corrupted, exploited, and affected ecosystem. Approximately 60% of the world's population, or nearly 3 billion people, live on or within 100 kilometres of the sea. India has a coastline length of 7,516.6 km (world bank., 2022). Pollution has become one of the most serious challenges in the modern environment. The definition of “pollution” was developed by the United Nations Educational and Cultural Organization (UNESCO), Group of Experts on the Scientific Aspects of Marine Pollution (GESAMP) “The introduction by man, directly or indirectly, of substances of energy into the marine environment resulting in such deleterious effects as harm to living resources, hazards to human health, a hindrance to marine activities including fishing, impairment of quality for use of seawater and reduction of amenities”(Dr Tanmoy Rudra., 2019). Plastics are one of the major pollutants in the marine ecosystem. Global plastics production accelerated from a million metric tonnes in the 1950s to 459.75 million metric tons in 2019. (Chibuisi Gideon Alimba and Caterina Faggio., 2019)

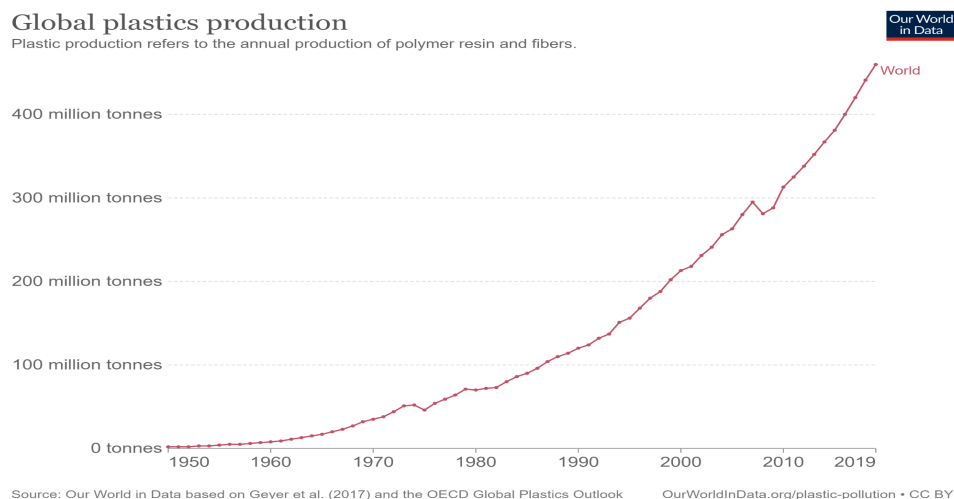


FIG 1.1 - Global plastics production (1950 - 2019)

1.1 PLASTICS:

Plastics are synthetic organic materials that can be formed into an array of shapes, films, or filaments by compressing, stiffening, or moulding. Leo Baekeland coined the term 'Plastics' and also discovered the first fully synthetic plastic *Bakelite*. The word plastic comes from the Greek word *plassein* which means to grow or form. Plastic is advancing into various forms, and several countries are starting to outlaw plastic due to its toxic nature. The toxic nature of plastics is seen mostly in micro and nanoplastics (Roland Geyer., 2020). Macro and mesoplastics do not cause any types of diseases, but they break down into smaller particles like micro and nano by fragmentation or degradation. The degradation of plastics is chemically, physically, or biologically induced. Biodegradable plastics are a type of recently discovered plastic that is rising in popularity since it generates less pollution and is readily biodegradable (Science Museum., 2020)

1.1.1 History of plastics:

Alexander Parks, an English metallurgist, and pioneer invented thermoplastics in 1850, which are a family of plastics which melt when subjected to heat and toughen once cooled. Parkes patented the item as *Parkesine* and presented it during *the Great Exhibition* of 1862 in London. *Parkesine*, however, did not find any financial prosperity. After researching with *Parkesine*, American inventor John Wesley Hyatt invented a means of producing solid, stable nitrocellulose, which he patented in the USA around 1869 as a *Celluloid*. *Celluloid* became the first commercially successful man-made polymer. Leo Baekeland, a Belgian-American chemist who devised the first thermoset after the discovery of *celluloid*, applied for patents on his groundbreaking creation, which he termed *Bakelite*, in 1907 in the USA and several other countries. *Bakelite* and *celluloid* are referred to as the "Dawn of the plastic age". However, given that they can't compete with contemporary plastics, *celluloid*, and *Bakelite* are now nothing more than collector's items. German scientist Eugen Baumann unintentionally discovered polyvinyl chloride, the origin of today's modern plastic, in 1872 (PVC). PVC is still manufactured in large quantities and is utilised widely to meet demand (Roland Geyer., 2020).

1.1.2 Effects of plastic pollution:

Plastic debris has a detrimental effect on both land and water ecosystems. The primary pollutants enter the marine environment in a broad range of ways, both on land and at sea. All ocean basins are economically and commercially significant. According to their sizes (Mesoplastics, microplastics, macroplastics, and nano plastics), the quantity of accumulated plastic in the marine environment is tracked (G G N Thushari and J D M Senevirathna., 2020)

1.1.3 Different types of plastics:

Plastics can be distinguished in two circumstances: one by their size, and the other by whether they melt when heated. Plastic is divided into four different categories based on its size mesoplastics, macroplastics, microplastics, and nanoplastics. The second distinction is whether or not they melt when heated. 1) If the plastic melts then they are divided into polyethylene (PE), polypropylene (PP), polyvinyl chloride (PVC), polyethylene terephthalate (PET), polystyrene (PS), and polyamide (PA), they are among the most well-known and extensively used plastics. They can also be found in thermoplastic. 2) Thermoplasts, the other group of polymers, are three-dimensional irreversible networks as opposed to linear or branched chains. Plastics derived from bio-based feedstock are less hazardous and economically sustainable (Roland Geyer., 2020).

Macroplastics(MaP) - are generally larger, and they are more than 5 mm in size. They are characterised as both primary and secondary plastics. Macroscopic pathways enter the environment through land- and ocean-based entryways. Ships are the primary source of macroplastic pollution in the oceans. Entanglement of organisms in MaP can result in drowning, suffocation, laceration, decreased fitness, lowered ability to hunt, or an increased likelihood of being caught. Degradation and fragmentation of MaP occur particularly in coastal areas where UV radiation and mechanical stress caused by wave movement are particularly high (Luís Gabriel A. Barboza *et al.*, 2019)

Mesoplastics - are large plastic components, as well known as virgin resin pellets, that generally vary in size from 5-100 mm. Meso Plastics are prevalent in the oceans; they

are established when macroplastics deteriorate into smaller particles, so they are macroplastic residues. They jeopardise marine life by acting as vectors for transporting toxic chemicals (Simone Lechthaler *et al.*, 2020)

Microplastics - typically range from 1 to 500 μ m. Microplastics emerge in a variety of shapes and sizes, including spheres, fragments, and fibres. Microplastics enter the body of an organism and cause problems, resulting in biodiversity loss. Microplastics and polymer additives are found in both industrial and domestic wastewater (Robert C. Hale *et al.*, 2020)

Nanoplastics - are plastic nanoparticles, which are any synthetic or semi-synthetic organic polymers with measurements anywhere from 1 and 100 nm. Nanoparticles interact with living organisms as free nano plastic particles or aggregates. Aquatic accumulation of primary and secondary nanoplastics poses a threat to the health of invertebrates and higher-level organisms which depend on the invertebrates for food. Nanoplastics trigger physiological distress, metabolic disorders, growth impediments, hindered autotrophic capabilities, diminished reproductive fitness, and early mortality (Karin Mattsson *et al.*, 2018)

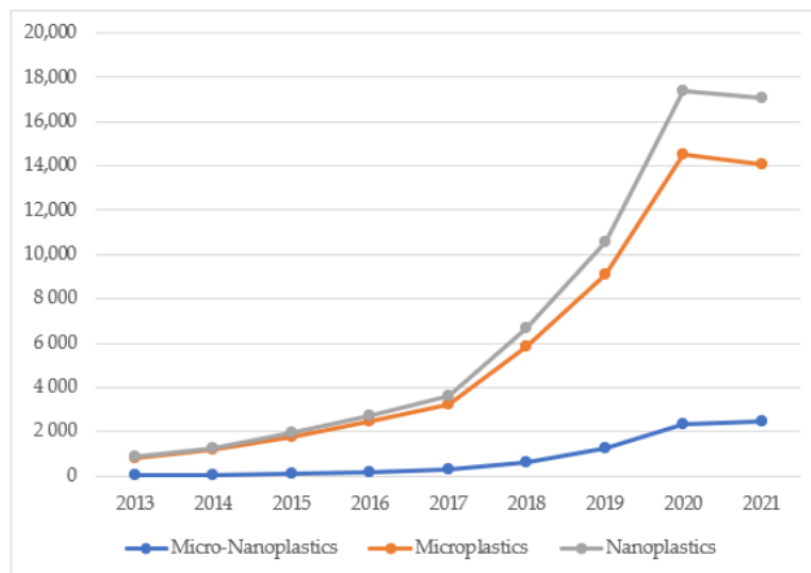


FIG 1.2 - Micro- nanoplastics, Microplastics and nanoplastics increase in the ocean (2013 - 2021)

Since our research is primarily focused on microplastics, we will provide a brief understanding of the study.

1.2 MICROPLASTICS:-

Microplastics are degraded forms of macroplastics with diameters greater than 500 mm. Microplastics are found in the human body, the soil, the air, marine animals, and the pharmaceutical and cosmetic industries. Microplastics enter the environment from a wide range of sources. 1)Wastewater from industries includes microplastics since many companies illegally discharge their effluent into rivers, and these microplastics are transported from the river to the ocean. 2)Macroplastic degradation - Macroplastics are both deliberately and unintentionally released into the water and when they are exposed to powerful wave currents, they deteriorate into smaller particles that cannot be detected with the naked eye; such particles are known as microplastics. In 2014, the accumulation of microplastic particles was estimated to be between 15 and 51 trillion particles, with a total weight of 93-236 thousand metric tonnes. The number of species affected by microplastics generally rises as the number of microplastics in the ocean increases. Aquatic species can come into contact with plastics in a variety of ways, including entanglement, ingestion, and interaction. Microplastics are of two types 1)Primary microplastics - Primary microplastics are plastics that have been manufactured to be microscopic. 2)Secondary microplastics - Secondary microplastics appear to be tiny plastic fragments developed by the breakdown of larger plastic debris, both at sea and on land (Marina Cerasa *et al.*, 2021)

1.2.1 Impacts of microplastics in the marine environment:-

The possibility that microplastics threaten biota is acquiring scientific attention, as their small size makes them accessible to a diversified range of marine organisms. Lower-trophic level organisms are extremely vulnerable to microplastics since many are indiscriminate feeders with a limited ability to discern between plastic particles and food. Microplastics, like macroplastics, may pose a mechanical hazard to small animals once ingested. Plastic fragments may block feeding appendages, impede food passage

through the intestinal tract, or cause pseudo-satiation, resulting in reduced food intake (Emma L Teuten *et al.*, 2007)

1.3 OIL SPILL:-

Pollution from petroleum or petroleum-related products has a considerable impact on marine biodiversity. Damage, transportation accidents, and a wide assortment of other mining and industrial activities all make a significant contribution to marine fuel spills. Researchers and environmental activists are extremely worried about marine habitat pollution. This is due to the severe implications of oil spills on aquatic life as well as on individuals whose livelihoods rely on the exploitation of sea resources (Zhendi Wang *et al.*, 1999)

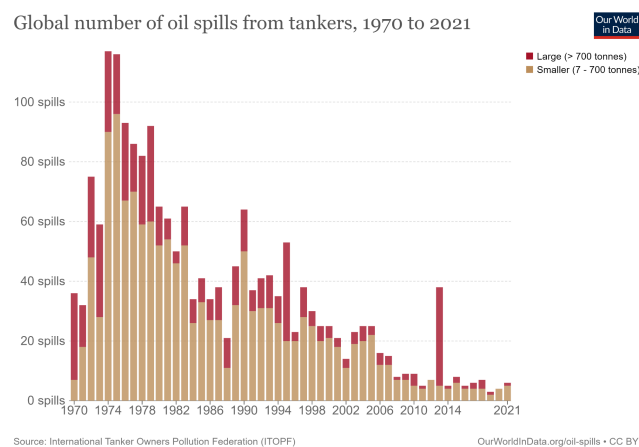


FIG 1.3 - Global no.of oil spills from tankers (1970-2021)

1.3.1 Characteristics of petroleum and crude oil:-

Petroleum is a complex combination of countless different organic materials established from various organic substances chemically converted over extended periods under widely different geological conditions. Crude oil is mostly made up of carbon and hydrogen, but it also incorporates minute quantities of sulphur, oxygen, and nitrogen, as well as metals like nickel, vanadium, and iron. Since these factors are extremely varied, there seem to be distinct chemical differences between oils. This variability in chemical composition results in distinct chemical "Fingerprints" for each oil, which can be utilised

to identify the source(s) of the oil spill. When marine animals ingest oil hydrocarbons, they travel to the liver and release toxic enzymes that are potentially lethal to the organism (Ismail M.K. Saadoun *et al.*, 2014).

1.3.2 Major oil spills related to the marine environment:-

Major oil spills in the world - Marine oil spillage, in particular, has evolved into a category of man-made catastrophes that have significant consequences for humans' ecological safety, economic benefits, and public safety, as well as ruining the marine ecosystem... The accepted average sample size for an oil spill is approximately 700 tonnes (5,061 barrels). During the 1990s, the average number of large spills per year was approximately one-third of what it had been in the 1970s. When considering the amount of oil spilt, 1,133,000 and 213,000 tonnes of oil were lost in the 1990s and 2000s, respectively. Between 2010 and 2013, 22,000 tons of oil were lost (Hong Mei and Yanjie Yin., 2009).

TABLE 1.4 - No.of oil spills from 7 tonnes, 1970-2013

Year	7-700 tonnes	>700 tonnes	Quantity Spilled x 10 ³ tonnes
1970-1974	189	125	1114
1975-1979	342	117	2012
1980-1984	221	41	570
1985-1989	124	48	513
1990-1994	165	48	907
1995-1999	108	25	194
2000-2009	182	-	213
2010-2013	28	-	22



FIG 1.5 - Location of top 20 major oil spills

Major oil spills in India - During the southwest monsoon months (June-September), when the cross-shore surface current begins to develop an easterly shoreward component, the Indian west coast is especially vulnerable to oil accidentally or covertly released in the Arabian Sea. The reported side effects of an oil spill on rocky intertidal macrofauna include oil asphyxiation, disruption of juvenile settlement, alteration in recruitment, growth, and community structure, reduction in species diversity, and extinction of sensitive species.

Around 4:00 a.m. on January 28th, 2017, two cargo ships, BW Maple, a liquid petroleum gas carrier, and MT Dawn Kanchipuram, a lubricant oil carrier, collided about two miles from the Ennore Kamarajar shipping terminal, which is located about 10 miles north of Chennai city in South India ($13^{\circ}13'41.4''\text{N}$ $80^{\circ}21'48.0''\text{E}$). The MT Dawn Kanchipuram was carrying approximately 32,000 tonnes of heavy bunker fuel oil when the accident occurred, and approximately 75 metric tonnes of the oil was spilt directly into the Bay of Bengal. Within 48 hours, the oil spill contaminated about 25 miles of coastline stretching from Chennai's northern suburb of Ennore to the southern suburb of Thiruvananthapuram (Soniya Sukumaran *et al.*, 2014)

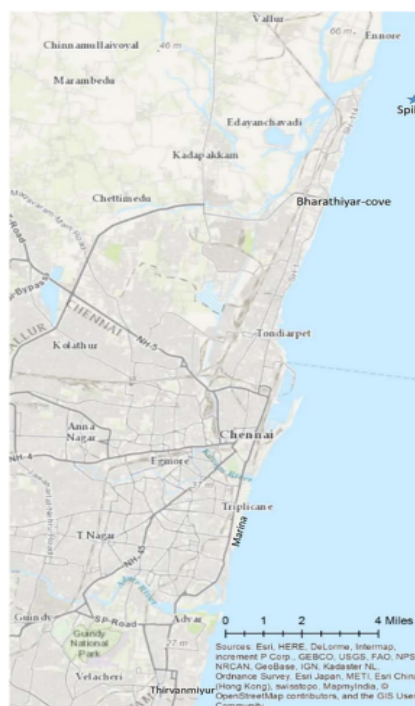


FIG 1.6 - 2017 Chennai oil spill location.

1.3.3 Impacts of oil spill on marine biodiversity:-

Several factors influence the consequences of the oil spill. Some compounds are toxic and harm delicate tissues, while others harm the eyes, nasal cavities, and other mucous membranes. To assess the consequences of oil exposure, it is necessary to understand the properties of petroleum, its composition, and how it enters the marine environment (Paul F Kingston *et al.*, 2002)

1.3.4 Impact of oil spills on planktonic community:-

Zooplankton is an especially important food source for aquatic organisms. Ingestion of oil by these organisms frequently results in death, while survivors frequently exhibit developmental and reproductive abnormalities. Many factors influence the lethal and sublethal effects of oil on marine zooplankton, including exposure time, oil type and concentration, salinity, temperature, food availability, and organism size and life history stage. Some zooplankton (e.g. Dinoflagellates, gelatinous doliolids, copepods) ingest oil and egest oil in faecal pellets, which may be ingested by other particle-feeding zooplankton (Jeff Chen and Michael S Denison., 2011).

1.3.5 Oil toxicity:-

The impact of oil on marine organisms ultimately depends on the fate of the oil. Oil toxicity depends on a multitude of factors, including the oil composition and characteristics (physical and chemical), condition (i.e. weathered or not), exposure routes and regimen, and bioavailability of the oil. One major effect of oil is narcosis, a reversible anaesthetic effect caused by the oil partitioning into cell membranes and nervous tissue, causing central nervous system dysfunction. Oil enters an organism by two main routes of exposure: through direct contact with the skin or by ingestion and/or inhalation. Toxicity resulting from oil/PAH ingestion occurs in all organisms and results from the action of liver enzymes attempting to degrade the oil PAHs (Jeff Chen and Michael S Denison., 2011).

1.4. BRINE SHRIMP (*Artemia salina*):-

The brine shrimp *Artemia* is a cosmopolitan crustacean that colonizes hypertonic media, considered lethal for the majority of aquatic species, varying from 9 ppt (parts per thousand) to 340 ppt. Depending on environmental conditions, this small crustacean can breed either ovoviviparous - producing free-swimming larvae “nauplii” or oviparous - producing encysted embryos “diapause cysts” enclosed within a chitinous shell. *Artemia nauplii* present an excellent live food item in larviculture due especially to its small size that matches perfectly with mouth-gape sizes of the first larval life stages of cultured species (Imene Sellami *et al.*, 2021).

1.4.1 Morphology:-

Artemia is a primitive arthropod with a segmented body to which are attached broad leaf-like appendages called thoracopods. Their length is ~8–10 mm for the adult male and ~10–12 mm for the female. The body is divided into the head, thorax, and abdomen. The head consists of one prostomial and five metameric segments which bear in order the median and compound eyes and labrum, first antennae, second antennae, mandibles, first maxillae and second maxillae (J. Dhont *et al.*, 2013).

1.4.2 Taxonomy:-

The genus *Artemia* (Crustacea, Brachiopoda, Anostraca) comprises several sexually reproducing species (‘bisexual species’) and several parthenogenetically reproducing populations. The identification of bisexual *Artemia* species has therefore been established by cross-breeding tests, morphological and morphometrical differentiation, cytogenetics, and allozyme studies; presently, increasing importance is being given to nuclear and mitochondrial DNA analysis, including sequencing (J. Dhont *et al.*, 2013).

1.4.3 Ecology:-

The brine shrimp *Artemia* is a zooplanktonic organism found globally in hypersaline habitats such as inland salt lakes, coastal salt pans, and man-managed salt works. *Artemia* is exceptionally adapted to such extreme environments, due to its unique osmoregulatory capacity and its capacity to synthesise highly efficient haemoglobins.

Being extremely osmotolerant, brine shrimp survive in environments with salinities ranging between approximately 10 and 340 g/L with diverse ionic composition and temperature regimes (Edis Koru., 2022).

1.4.4 Reproduction:-

Artemia reproduces in two modes, involving either nauplius (ovoviviparous) or cyst (oviparous) production, depending on the prevailing ecological conditions. Ovoviviparity occurs under favourable ambient conditions: eggs (fertilised following a mating in the case of bisexual species or non-fertilized in the case of parthenogenetic females) produce free-swimming larvae ('nauplii') released by the mother. On the other hand, oviparous reproduction occurs under unfavourable conditions, usually characterised by factors such as high salinity, low oxygen levels, temperature stress, food depletion, etc. Both oviparity and ovoviviparity are found in all *Artemia* strains, and female individuals can switch from one mode to the other between two reproduction cycles. Under favourable ecological conditions, *Artemia* can live for several months, growing from nauplius to adult in only eight days and reproducing at up to 300 nauplii or cysts every four days (Edis Koru., 2022).

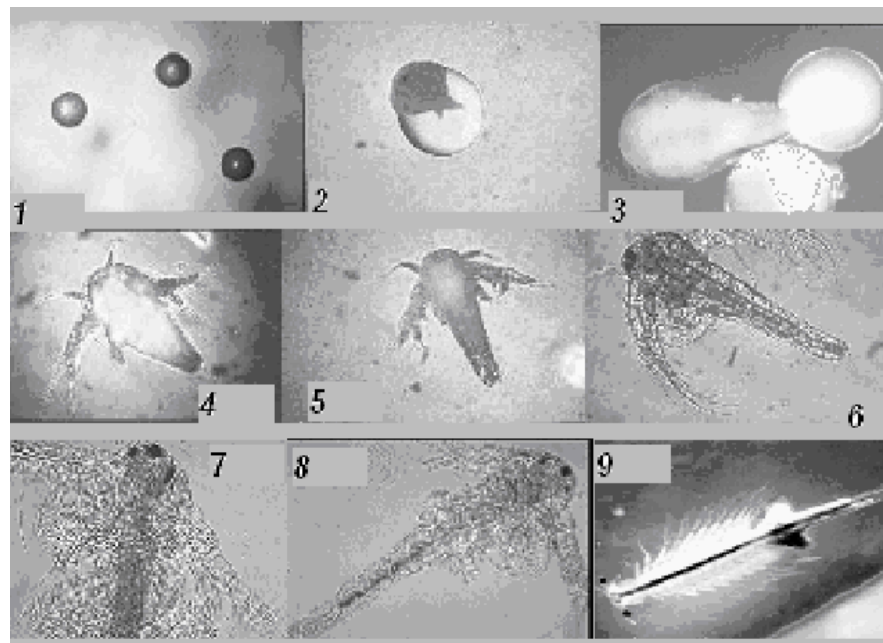


FIG 1.7 - Steps in the life cycle of Artemia.

1.4.5 History of *Artemia*:-

The status of *Artemia* as an economic commodity dates back to the 1930s when some investigators adopted it as a convenient replacement for the natural plankton diet for fish larvae thus releasing the first breakthrough in the culture of commercially important fish species. In the 1950s, *Artemia* cysts were still predominantly marketed for the aquarium and pet trade; there were only two commercial sources: the coastal salt works in the San Francisco Bay (California, USA) and the Great Salt Lake (GSL) (Utah, USA). The dramatic impact of the cyst shortage in the mid-70s on the expanding aquaculture industry invigorated research on the rationalisation of the use of *Artemia* and the exploration of new cyst resources. Along with the exploitation of natural resources, intensive cyst production in solar saltworks comprises an important market share in terms of high product quality and its importance in sustainable aquaculture development in many countries in the south. Often this production is carried out seasonally (e.g. in monsoon Southeast Asia) in areas where there is no natural occurrence of *Artemia* (J. Dhont *et al.*, 2013).

4.6 *Artemia* genus in ecotoxicology:-

The early 1980s witnessed large developments in Ecotoxicology as an autonomous science in the modern scientific world. New concepts were brought to actual research, as a part of an effort to adapt cost-effective procedures, which comply with accuracy, reliability, and ecological relevance. An international inter-calibration exercise stated that 87% of all the involved participants were able to complete the standardised test with *Artemia*, and evaluated the test results as simple to interpret, reliable and accurate. The present status of Ecotoxicology shows a thorough use of short-term acute cyst-based toxicity screening assays. The use of cysts is highly advantageous whenever needs concerning screening toxicity tests arise. *Artemia* nauplii have also been used in marine toxicity testing in coastal areas and for establishing alternative toxicity tests (Edis Koru., 2022).

1.5 MICROBIAL COMPOSITION:-

In 1933, American scholar Seale discovered that *Artemia* nauplii are the surest bet for aquatic animals since the nauplii stage of *Artemia* contains a high level of proteins and unsaturated fatty acids, that can fulfil the nutritional requirements of crustaceans and fish larvae. As a result, the comparative study of the diversity of *Artemia* nauplii symbiotic microorganisms has a very key reference value for biological bait microorganisms to regulate seedling intestinal health and purify aquaculture water quality.

In a study conducted by (Yang Xu., 2022) the quality and symbiotic microbial composition of nauplii in different areas were studied. Their results were that there were a total of 45 phyla, 111 classes, 276 orders, 457 families, 872 genera, and 1,577 species. In the phylum level, the majority of the sequences belonged to nauplii *Firmicutes* (37.760- 51.52%), *Proteobacteria* (33.11-42.01%), *Bacteroidata* (3.26-15.41%), *Actinobacteria* (0.51-7.20%), and *Acidobacteria* (0.18-1.86%) respectively. At the genus level, *Bacillus* (16.25–25.56%), *Pseudomonas* (11.73–20.34%), *Exinguobacterium* (3.61-21.21%), *Pontibacter* (2.71-12.39%), *All Rhizobium -Neorhizobium-Pararhizobium-Rhizobium* (0.18-11.66%) are dominant. Other dominant bacterias include *Mycoplasma*, *Microbacterium*, *Halomonas*, and *Psychrobacter* (Yang Xu., 2022).

CHAPTER - 2

REVIEW OF LITERATURE

Recently, research has begun to focus on the effects of microplastics on zooplankton, particularly brine shrimp. Microplastics are microscopic pieces of plastic debris that have been found in the marine environment and are considered major environmental pollutants. Research has found that microplastics can be ingested by brine shrimp, leading to changes in their behaviour, decreased feeding, and increased mortality. Microplastics have also been found to accumulate in the organs of brine shrimp, leading to decreased growth and reproduction. Hence it is crucial to discuss the studies conducted on the above statement.

Global plastics production increased from 1.5 million tons in the 1950s to 335 million tons in 2016. Plastics take plenty of time to degrade, but often, through different processes, they fragment into microplastics and nanoplastics, which are ubiquitous pollutants in the marine environment. The following research looked into the toxicological effects of microplastics in marine environments. They have noted that microplastics have a greater toxicity effect on marine organisms since they can observe organic contaminants, metals, and pathogens which are transferred to marine organisms through ingestion. This study has concluded that with increasing human activities along with poor sanitation, there will be an increase in microplastics in the marine environment. Cetaceans, sea reptiles, seabirds, and marine fishes are highly vulnerable to entanglement in plastic debris with significant widespread suffering; including asphyxiation by drowning, starvation, restriction to movements, injuries due to abrasions, and mortality. They also cause abnormalities in the gastrointestinal tract of the organism which leads to the induction of oxidative stress, immunological responses, genomic instability, disruption of the endocrine system, neurotoxicity, reproductive abnormalities, embryotoxicity, and trans-generational toxicity (C.G. Alimba and C. Faggio., 2019).

Microplastics have a drastic effect on zooplankton which is an important element of the aquatic food chain. Since our study is focused on *Artemia* or commonly known as Brine shrimp we will give an elaborate account of the toxicity of microplastics in *Artemia*.

According to a study by (L. Kim *et al.*, 2021), microplastics have caused significant gastrointestinal tract damage, which in some organisms leads to mortality. The methodology for this research is that *Artemia* is first injected with microplastics and afterwards stained with a dye (Brilliant blue FCF). Increased permeability in the intestinal layer was commonly observed in the head area. In the instance of minor damage, the dye managed to pass through the gut layer in tiny portions until the entire dye content was eliminated from the body.

The persistent rise of microplastic pollution in the aquatic ecosystem has been a major concern worldwide. Although studies on microplastics have been ongoing for a while, there is still much to learn about their effects on human health and marine life. The study done by (Hye-Jin Eom *et al.*, 2020) exposed *Artemia* to 4 different types of polystyrene microplastics to study their effects on the test organisms. To measure microplastic effects, several physiological and biochemical parameters such as ingestion and egestion, and stress biomarkers such as enzymatic activities of catalase (CAT) and superoxide dismutase (SOD) activity are done to measure microplastic effects. On close examination, there was clear ingestion and egestion in *Artemia*. The ingestion of microplastics by the organism leads to cell stress which eventually causes the mortality of brine shrimp. Hence, they concluded that exposure to microplastics in the early stages of brine shrimp causes acute and oxidative cell stress.

Microplastics' molecular toxic effects on brine shrimp remain an enigma. A thorough explanation of the acute and chronic toxicity resulting from exposure to polystyrene microplastics in brine shrimp at varying concentrations is presented in the study by (Thodhal Yoganandham Suman *et al.*, 2020). The outcome of their study revealed that the brine shrimp's ability to survive had not been significantly harmed, but reactive oxygen species (ROS) had increased concentration-dependently, and histopathology results had revealed deformation of midgut epithelial cells. This study provided comprehensive sequencing data from transcriptome profiling to determine the molecular toxicity of polystyrene microplastics in brine shrimp in addition to highlighting the harmful effects.

Microplastics are further fragmented to form nanoplastics, which have also emerged as a major issue. Ignacio Martinez-Alvarez *et al.*, 2022 conducted the acute toxicity of polystyrene microplastics and nanoplastics alone and in combination with Benzo(a)Pyrene in Brine shrimp larvae and Zebrafish embryos. The following is a summary of their study methodology. 1) Brine shrimp were exposed to pristine plastics with no significant effect on the organism's survival; however, exposure to a combination of microplastics and B(a)P resulted in acute toxicity. 2) Only the highest concentrations of MPs-B(a)P and B(a)P in zebrafish caused the severe malformation. NPs were noticed in the body surface and digestive tract of brine shrimps and faeces. NPs were found in zebrafish's eyes, yolk sac, and tail. Only brine shrimp were subjected to microplastics. As a direct consequence, nanoplastics entered and spread into the zebrafish embryo and pristine nanoplastics, as well as microplastics, were successful vectors of B(a)P to brine shrimp and zebrafish embryos. Particle size was crucial in explaining the toxicity of plastics-B(a)P. This study is an excellent example of plastic toxicity on aquatic organisms combined with organic pollutants such as Benzo(a)Pyrene.

The effects of oil spills on zooplankton, specifically brine shrimp, have been extensively studied in the past decades. Several studies have demonstrated that oil spills can cause significant damage to zooplankton populations and disrupt the natural balance of the marine environment. The most immediate effect of a spill is the physical contact between oil and zooplankton. The oil can coat the outside of the brine shrimp, blocking their respiratory pores and leading to suffocation. In addition, the oil can disrupt the brine shrimp's natural behaviour, causing them to reduce or completely cease feeding and reproduction. In addition to direct physical contact, oil spills can also cause significant damage to zooplankton indirectly. Oil spills can cause oxygen depletion in the water, leading to a decrease in zooplankton populations due to decreases in available food sources. Oil spills can also release toxic substances into the water, which can be absorbed by the brine shrimp and cause a decrease in fertility and a decrease in the health of the population. Overall, oil spills have been found to negatively impact brine shrimp populations, leading to decreases in population size, fertility, and health. To

reduce the damage caused by oil. A few studies which elaborate on this subject are detailed below.

Polycyclic aromatic hydrocarbons (PAHs) are organic compounds that are widely distributed in the air, water, and soil. The amount of PAHs derived from fuels and incomplete combustion processes is increasing. In the aquatic environment, oil spills are a direct source of PAH pollution and affect marine organisms. Accidental oil spills can seriously impact the marine environment toxicologically. The study conducted by Honda and Suzuki, 2020 elaborates on the toxicity of polycyclic aromatic hydrocarbons in aquatic animals. They also describe the PAH toxicities and related bioaccumulation properties in aquatic animals, including invertebrates. Microplastics have been the most concerning environmental problem in the aquatic ecosystem, and the vector effect of microplastics on lipophilic compounds is an emerging environmental issue. Hence, this study describes the correlation between PAHs and microplastics. The conclusion of this study is as follows 1) Oil contains several kinds of PAHs. 2) low-molecular-weight compounds, such as naphthalene and three-ring PAHs, accumulate in fish and invertebrates. 3) The PAHs derived from the aquatic environment are accumulated and are toxic to fish and invertebrates. 4) *Artemia salina* tends to be more sensitive to pyrene and the LC50 test determined this. Alkyl-PAHs is the predominant form of PAHs in crude oils and is supposed to demonstrate different toxicities compared to non-alkyl PAHs still there are only a few studies related to this topic. The study done by Yi Cong *et al.*, 2021 observed and correlated the lethal, behavioural, growth and developmental toxicities of three alkyl-PAHs addressed and compared the lethal, behavioural, growth, and developmental toxicities of three alkyl-PAHs (3-methyl phenanthrene (3-mPhe), retene (Ret) and 2-methyl anthracene (2-mAnt)) and non-alkyl forms (phenanthrene (Phe) and anthracene (Ant)) using *Artemia* as test organism. Benzo-a-pyrene (BaP) was selected as a reference toxicant for the comparison with the above alkyl-PAHs and non-alkyl PAHs. Results showed that for all tested endpoints, *A. parthenogenetica* nauplii had the highest sensitivity to BaP while Ant had no significant effect on nauplii survival or development within given concentrations. A higher acute toxicity of alkyl-PAHs than their non-alkyl forms was observed. Organisms that were exposed to PAH treatments had decreased body lengths compared to control, whereas nauplii that

were exposed to Bap, Phe, and Ret had a reduction in the instar stage. Hence, this study has emphasised the sensitivity differences of *Artemia* nauplii to selected alkyl PAHs and non-alkyl PAHs and confirmed the application of lethal, behavioural, and growth indicators in the toxicity evaluation of selected PAHs. In addition to that, a strong combination between body length and instar of *Artemia parthenogenetica* nauplii was observed for each PAH exposure, suggesting that body length can be representative of both growth and developmental indicators during biological monitoring of PAH pollution in the marine environment.

To understand the effects of oil spills in the zooplankton community we consider the following study by Rodrigo Almeda *et al.*, 2013 into consideration. Rodrigo and his colleagues conducted ship-, shore- and laboratory-based crude oil exposure experiments to investigate the following points. 1) The effects of crude oil on survival and bioaccumulation of polycyclic aromatic hydrocarbons (PAHs) in mesozooplankton. 2) The lethal effects of dispersant and dispersant-treated oil on mesozooplankton. 3) The influence of UVB radiation/sunlight exposure on the toxicity of crude oil to mesozooplankton. 4) The role of marine protozoans in the sublethal effects of crude oil and the bioaccumulation of PAHs in the copepod *Acartia tonsa*. The exposure of mesozooplankton to oil showed that when the concentration of oil increases the mortality of mesozooplankton also increases. UVB radiation increased the lethal effects of dispersed crude oil in mesozooplankton communities by 35%. Bioaccumulation of five PAHs namely fluoranthene, phenanthrene, pyrene, chrysene, and benzo[b]fluoranthene in both mesozooplankton communities and the copepod *A. tonsa*. The protozoans present in the ocean reduced accumulations of PAHs in the tissues and faecal pellets of *A. tonsa*. Hence, protozoa are important to reduce the sublethal effects of oil on *A. tonsa*. It is also critical in mitigating the harmful effects of crude oil exposure in copepods and the transfer of PAHs to higher trophic levels. The results of this study indicate that the negative impact of oil spills on mesozooplankton may be increased by using chemical dispersants and UV radiation, and it can be attenuated by oil-microbial food web interactions. They also concluded that both mesozooplankton and protozoans may play an indispensable role in the fate of PAHs in marine environments.

The microbial composition of *Artemia* has been studied extensively in recent years. Studies have revealed a wide variety of microbial species associated with shrimp, including bacteria, protists, and fungi. Bacterial species identified in *Artemia* include members of the genera *Pseudomonas*, *Acinetobacter*, *Staphylococcus*, and *Vibrio*. These bacteria are thought to be beneficial for the health of the shrimp, protecting them from disease and other environmental insults. Protists such as *diatoms*, *dinoflagellates*, and *ciliates* have also been found in *Artemia*, and play an important role in the maintenance of the shrimp's environment. Fungi, including yeasts, filamentous fungi, and oomycetes, have also been identified in *Artemia*, and are thought to contribute to the health and fitness of the shrimp by providing nutrients. Overall, the microbial composition of *Artemia salina* is diverse and dynamic. Some of the studies which elaborate on the microbes present in *Artemia* are as follows.

CHAPTER - 3

AIM AND SCOPE OF THE PRESENT INVESTIGATION

3.1 AIM:

To determine the impacts of the combined toxicity of polystyrene microplastics and diesel pollution in the marine brine shrimp (*Artemia salina*)

3.2 SCOPE:

- To conduct experimental toxicity of microplastics and diesel pollution in marine brine shrimp.
- Culture and maintenance of marine brine shrimp (*Artemia*).
- Preparation of toxicants.
- Determination of Lethal Concentration (LC₅₀) value.
- To perform the Probit analysis to determine the LC₅₀ value of the test organisms.
- Microscopic assessment of the organisms exposed to toxicants.
- Identification of microbial contamination in experimental groups.
- To analyse acute gut toxic exposure.

CHAPTER - 4

MATERIALS AND METHODS

4.1 TOXICANT MATERIALS:

Thermo Fisher Scientific, India, supplemented commercial Fluospheres™ Polystyrene Microspheres (catalogue number: F13080) with a diameter of 1µm, a concentration of 1×10^{10} beads/mL, a volume of 5 mL, and blue-green fluorescence at 430/465 nm. They were kept in a refrigerator (2-8 °C) and kept protected from light. Diesel was purchased from Indian Oil Corporation Ltd., a petrol bunk.

4.2 HATCHING OF THE TEST SPECIES:

The most widely used species used for ecotoxicological purposes is the *Artemia* species. The dry cysts for this study were purchased from Gold West Artemia (USA). Filtered Sea water (FSW) was used throughout the whole procedure. For decapsulation, they were treated with (FSW) and 2% Sodium Hypochlorite for 2 minutes each. The cysts were then transferred to a glass beaker containing 500ml of FSW for culturing. The culture was maintained with accurate temperature and pH throughout the hatching process. The seawater was maintained at a pH of 9 and a salinity of 30 ppt. The cysts were kept under a photoperiod of 12h light/ 12h dark. The 48-h nauplii were harvested and used for further experimental purposes.



FIG 4.1 - Hatching of Artemia cyst

4.3 TOXICOLOGY STUDY:

4.3.1 Toxicity media preparation:

Four different media were prepared for this study. The different media were prepared in four beakers A, B, C and D. FSW was used as the control medium. All the beakers contained 100 mL of FSW. Beaker A had sea water and Beaker B contained 10^4 particles/ml MPs. Beaker C contained 0.5µl of Diesel. Beaker D contained both MPs and diesel of the respective calculated rate. This is used to determine the combined toxicity of both toxicants on the *Artemia* nauplii. (Hye-Jin Eom *et al.*, 2020, Rodrigo Almeda *et al.*, 2013)

4.4 RANGE FINDING TEST:

The LC_{50} value was determined using five different concentrations of MPs (10^2 , 10^3 , 10^4 , 10^5 and 10^6). In every well of a 6-well plate, 10 mL of FWS was added and 30 individuals were added to each well. The results were checked after 24h and 48h. The survival rate of the nauplii was assessed using probit analysis. The LC_{50} value for diesel-exposed organisms was also determined. Five different concentrations (5µl, 10µl, 20µl, 50µl and 100µl) were used for this test.

4.5 PROBIT ANALYSIS:

Probit analysis is a statistical method for illustrating and analysing the association between a variable with a binary response (e.g., yes/no) and a number of predictor variables. To determine the mortality ratio of the toxicants microplastics and diesel, their concentrations were converted into log₁₀ concentrations. Finney's table was used for converting the mortality percentage into probit value. The curve was drawn with the log concentration on the X axis and the probit concentration on the Y axis.

4.6 MICROBIAL STUDY:

Artemia was collected in a 1.5µl centrifuge tube containing 500µl of Phosphate buffered saline (PBS) solution for this study. *Artemia* was ground until the organism's residues settled at the bottom. After they had settled, a further 500 µl of PBS was added.

Following the preparation of the solution, serial dilution was carried out in a sterile environment. Four different test tubes were chosen for serial dilution. The concentrations of these test tubes are 10^1 , 10^2 , 10^3 , and 10^4 . 1 ml of grounded *Artemia* mixture was transferred into each of the test tubes accordingly. Following serial dilution, a spread plate method was performed. For the spread plate concentration, 10^3 and 10^4 was chosen, and 100 μ l of the solution was transferred to a Petri plate containing solidified nutrient agar. After transferring into the Petri plate, the solution was extensively spread. When the spread plate was completed, it was sealed with parafilm and placed into an incubator.

4.7 COLONY MORPHOLOGY AND COLONY COUNTING:

The 24hr culture was analysed for the growth of microbes. The colonies were assessed and the morphology for different colonies was noted down. The morphology was evaluated based on shape, size, margin, elevation, appearance, optical property, texture, and pigmentation. The colonies were then used to perform the streaking method.

4.8 STREAK PLATING TECHNIQUE:

To acquire a pure culture from the above growth culture, the streaking method was followed. The streaking method was done for the 24-hour culture. The whole process was done inside a Laminar airflow chamber. The inoculum loop was heated until it was red hot. The continuous streaking method was done after the culture had been taken in the loop. The petri dish was sealed using parafilm and kept for further incubation.

4.9 GRAM STAINING:

A slide is taken and a drop of sterile water is kept in it. Using an inoculum loop, a small amount of culture is taken and smeared on the slide. The slide is then heat-fixed on the spirit lamp. All the above procedures were done inside a Laminar Airflow chamber. The gram staining procedure was done following this. The heat-fixed slides were taken and a few drops of crystal violet stain was added. It was kept for 1 min and was washed off with distilled water. Further, Gram's Iodine was added and was washed after 1 minute.

alcohol/acid solution was added to decolourize the primary stain. It was kept for 30 seconds and washed off. Finally, safranin was added as the counterstain for 1 min and it was also further washed off. This procedure was repeated for all the slides. The slides were then viewed under a Leica Light microscope. The images were viewed at 40x magnification. The Gram type and shapes of the bacteria were analysed.

4.10 MOTILITY:

Motility was determined using the hanging drop method. The bacteria were taken from the streak plates. It was inoculated in nutrient broth for 3 hours. The procedure was done after a growth in the inoculum tubes was observed. A few drops were placed on a concave slide and covered using a coverslip. The slides were placed under a Leica Light microscope and the movement was viewed at 10x magnification.

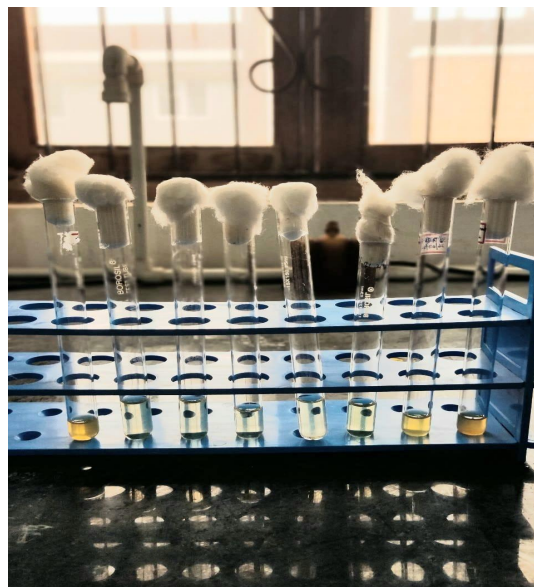


FIG 4.2 - Nutrient broth inoculated with 3hr streak plate culture for motility test

4.11 ACUTE GUT EXPOSURE ANALYSIS:

The acute gastrointestinal exposure technique was used to assess the damage to the organism's gut after exposure to the toxicity media. *Artemia* was acutely exposed to four different types of exposure media for 24 hours. The organism's gut was stained with 2µl of Coomassie Brilliant Blue stain to further expose it. The duration of incubation was two hours. A few drops of formaldehyde were used to fixate the organism after it was placed in a slide. Using a Leica Phase contrast microscope, it was analysed. The slides were viewed under a phase contrast microscope at 10x magnification.

CHAPTER - 5

RESULTS AND DISCUSSION

5.1 RANGE FINDING TEST (LC₅₀):

The survival rate for both the microplastic and diesel-exposed organisms was analysed. The number of organisms that survived was observed. The results were obtained and tabulated. The lethal concentration was assessed by the percentage of survival in each well. The lethal concentration of microplastic exposed organisms is given in Table 5.1 and the graph is plotted in Fig 5.2. Further, the LC₅₀ value for organisms exposed to diesel was also tabulated and plotted in a graph (Table 5.3, Fig 5.4). From the above results and graph, it can be seen that for microplastics the population reduces at 1,00,000 particles/ml at 48 hours and for diesel, it reduces at 1µl/ml at 48 hours.

TABLE 5.1 - The survival percentage of the *Artemia* population exposed to Microplastics.

Microplastics concentration

S.no.	Microplastic Concentration (Interpretation)	% survival	
		24 H	48 H
1)	N.C.	100	100
2)	T1 - 10 ²	100	90
3)	T2 - 10 ³	70	80
4)	T3 - 10 ⁴	60	70
5)	T4 - 10 ⁵	50	40
6)	T5 - 10 ⁶	30	4

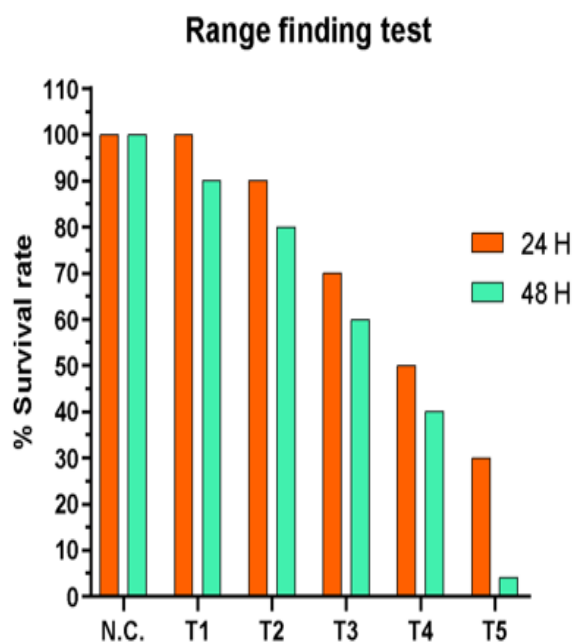


FIG 5.2: The graph plotted showing the survival percentage exposed to Microplastics.

TABLE 5.3 - The survival percentage of the Artemia population exposed to Diesel.

S.NO	DIESEL CONCENTRATION	% SURVIVAL	
		24 hr	48 hr
1)	NC	100	100
2)	T1 (0.5µl /ml)	100	100
3)	T2 (1µl /ml)	97	17
4)	T3 (2µl /ml)	90	0
5)	T4 (5µl /ml)	0	0
6)	T5 (10µl /ml)	0	0

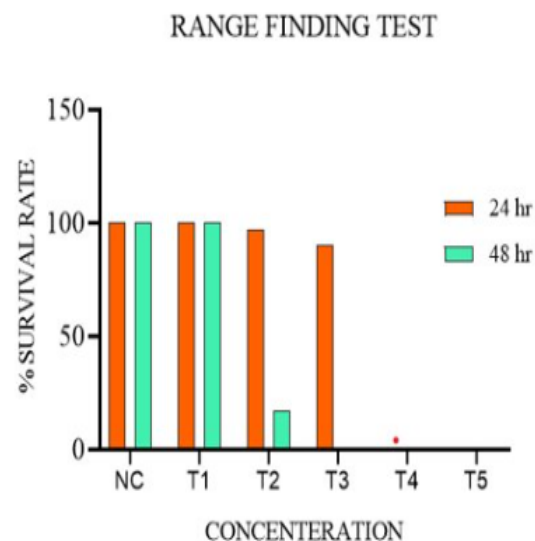


FIG 5.4: graph plotted to show the survival percentage of the Artemia population exposed to diesel

5.2 PROBIT ANALYSIS:

The results of the probit analysis were tabulated in Table 5.5. For both microplastics and diesel, the R^2 and standard error values were determined for 24 hours and 48 hours, respectively. The graph was plotted using Microsoft Excel and the results was obtained.

TABLE 5.5 - Probit analysis results. RE - Regression equation; SE - Standard error

SI NO	EXPOSURE PERIOD											
	MICROPLASTICS						DIESEL					
	24 hr			48 hr			24 hr			48 hr		
	RE	R^2	SE	RE	R^2	SE	RE	R^2	SE	RE	R^2	SE
1	$Y=0.609x + 2.204$	0.8958	0.4407	$Y=0.879x + 1.626$	0.8415	0.6965	$y = 5.769x + 2.386$	0.647	0.9212	$y = 4.879x + 4.175$	0.798	2.642

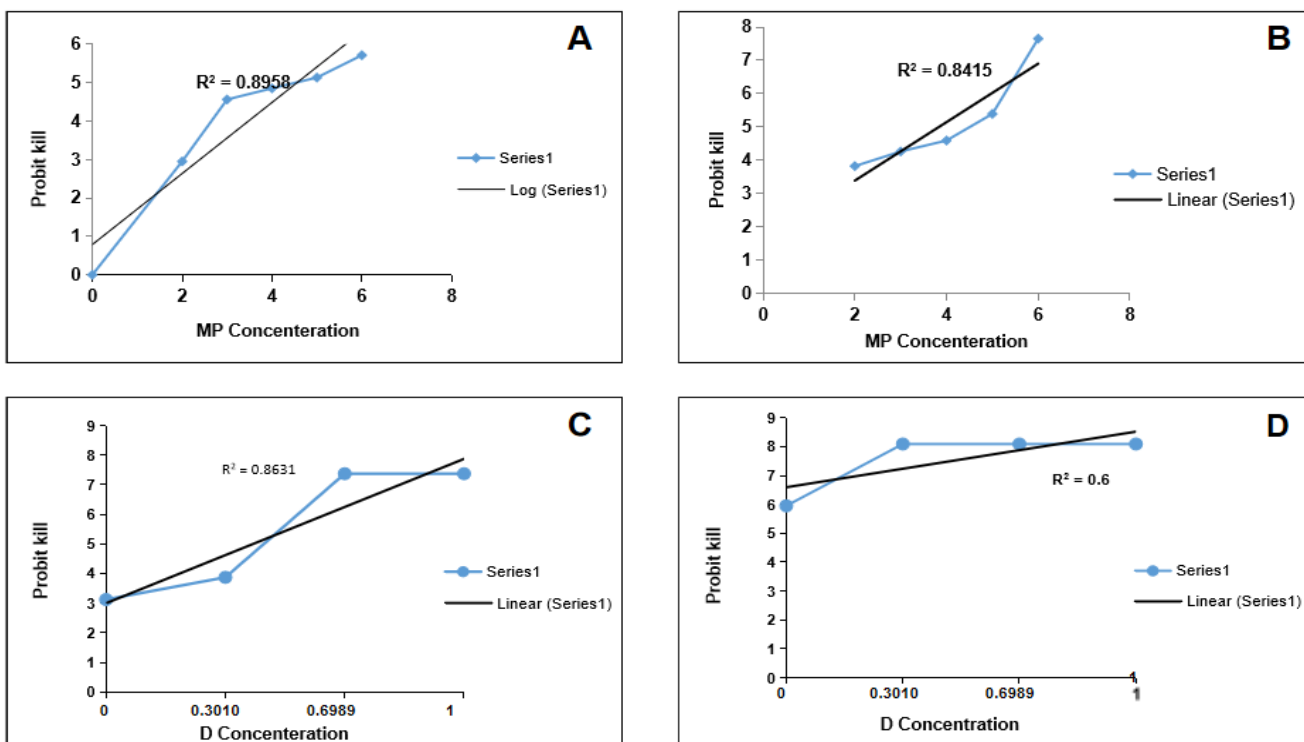


FIG 5.6 A - Probit graph for MP 24hr; B - Probit graph for MP 48hr; C - Probit graph for Diesel 24hr; D - Probit graph for diesel 48hr. The X-axis of the graph incorporates the concentration of MP and Diesel converted into log₁₀ value, whereas the Y-axis includes the probit value of test animal mortality percentage.

TABLE 5.7 - Probit analysis results for Microplastics

SI.NO	MICROPLASTICS			
	24 hr		48 hr	
	LOG ₁₀ (Conc)	Lethal concentration	LOG ₁₀ (Conc)	Lethal concentration
1	0	1	3.82	6606.9
2	2.95	891.3	4.26	18197.01
3	4.56	36307.81	4.59	38904.51
4	4.85	70794.6	5.39(LC ₅₀)	245470.9
5	5.13(LC ₅₀)	134896.3	7.65	44668359
6	5.71	512861.4		

TABLE 5.8 - Probit analysis for Diesel

SI.NO	DIESEL			
	24 hr		48 hr	
	LOG ₁₀ (Conc)	Lethal concentration	LOG ₁₀ (Conc)	Lethal concentration
1	3.12	1318.2	5.95	891250.9
2	3.87	7413.1	8.09	123026877.1
3	7.37	23442288.2	8.09	123026877.1
4	7.37	23442288.2	8.09	123026877.1

5.3 MICROBIAL STUDY:

The serial dilution method was done to reduce the number of colonies present in the sample. The concentration that was taken included 10^1 , 10^2 , 10^3 , 10^4 , 10^5 and 10^6 . The concentrations selected for spread plating were 10^3 and 10^4 . The results for the spread plates were observed after 24 hours.

5.4 COLONY MORPHOLOGY AND COLONY COUNTING:

The 24-hour spread plate of the various exposure media was summarized. The number of colonies present was enumerated. They were categorised in Table 5.7. The following morphological characteristics were used to evaluate the colonies: shape, margin, appearance, optical property, elevation, and texture. These morphological features were examined and tabulated in Table 5.7. There were four colonies in the control plates, two in the diesel plates, two in the microplastics plates, and four in the combined exposure plates when colonies were counted.

TABLE 5.9 - Colony morphology and colony count result

COLONY MORPHOLOGY	CONTROL				D		MP		D + MP			
	(1ST)	(2ND)	(3RD)	(4TH)	(1ST)	(2ND)	(1ST)	(2ND)	(1st)	(2nd)	(3rd)	(4th)
SHAPE	Circular	Circular	Filamentous	Circular	Matte	Matte	Matte	Irregular	Circular	Filamentous	Circular	Circular
MARGIN	Entire	Entire	Filamentous	Entire	Irregular	Irregular	Entire	Entire	Entire	Filamentous	Entire	Entire
APPEARANCE	Shiny	Dull	Dull	Shiny	Dull	Dull	Dull	Shiny	Shiny	Dull	Shiny	Dull
OPTICAL PROPERTY	Translucent	Opaque	Opaque	Translucent	Transparent	Translucent	Translucent	Opaque	Opaque	Opaque	Translucent	Opaque
ELEVATION	Flat	Convex	Raised	Flat	Flat	Flat	Flat	Elevated	Elevated	Flat	Flat	Flat
TEXTURE	Smooth	Smooth	Dry	Smooth	Smooth	Dry	Smooth	Smooth	Smooth	Dry	Smooth	Dry

5.4.1 Spread plate result:

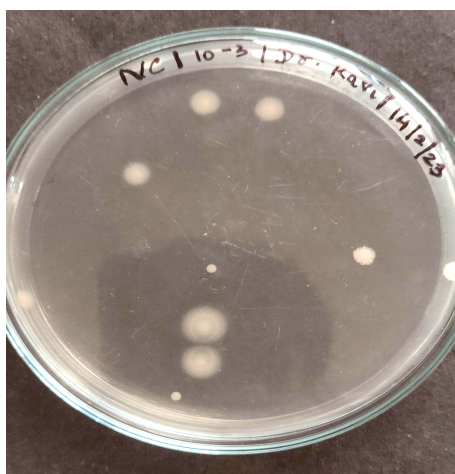


FIG 5.10 - Negative control

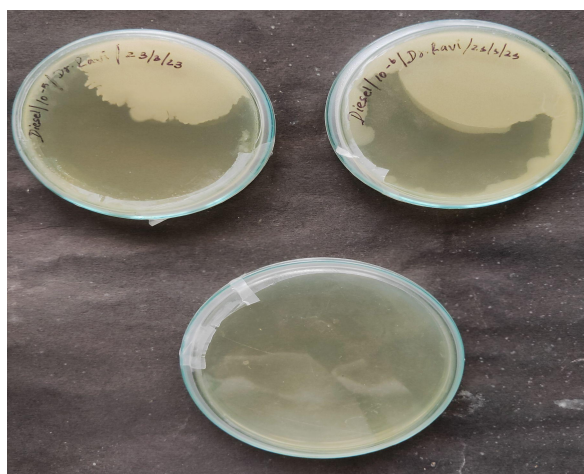


FIG 5.11 - Diesel 10⁴ & 10⁵

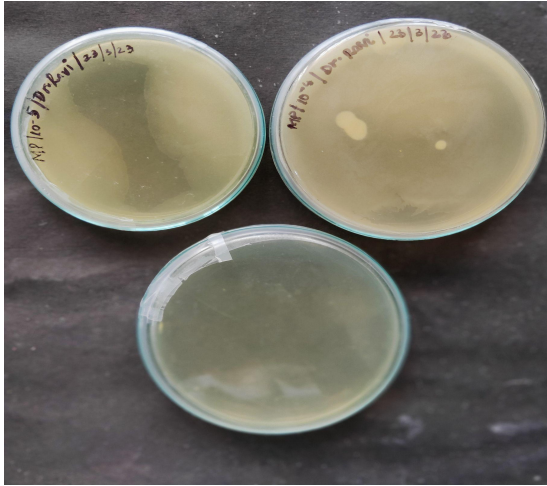


FIG 5.12 - Microplastics 10^5 & 10^6



FIG 5.13 - Diesel + Microplastics 10^4 & 10^5

5.4.2 Streak plate results:

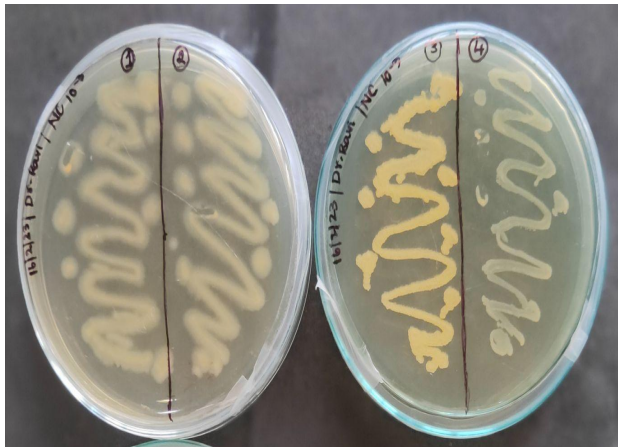


FIG 5.14 - Negative control

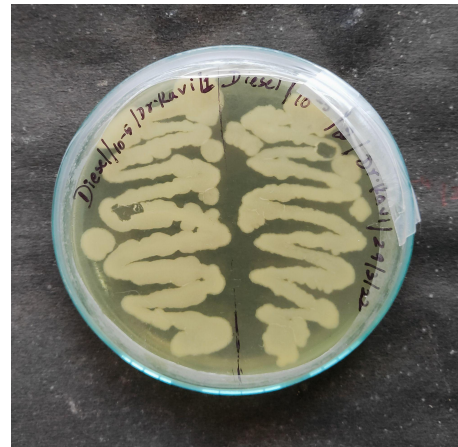


FIG 5.15 - Diesel

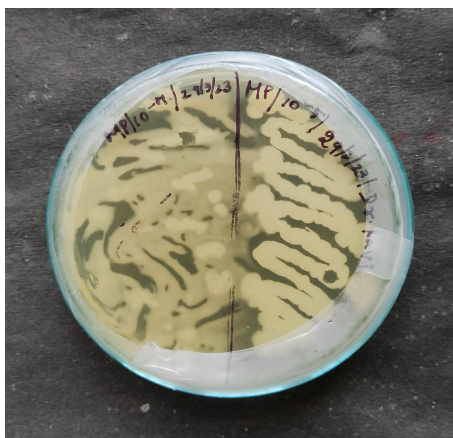


FIG 5.16 - Microplastics



FIG 5.17 - Diesel + Microplastics

5.5 GRAM STAINING:

The gram staining procedure yielded the following results; NC contained both Gram-positive and Gram-negative bacteria with rods, cocci in clusters and chains and mixed culture. Diesel, Microplastics and the combined D+MP contained Gram-positive bacteria with Rod-shaped and mixed culture bacteria.

TABLE 5.18 - Gram staining characteristic

MEDIUM	GRAM TYPE	SHAPE
NC	Gram- Negative	Rods
NC	Gram- Positive	Mixed culture
NC	Gram- Negative	Cocci in clusters
NC	Gram- Negative	Cocci in chains and clusters
DIESEL	Gram- Positive	Rods in chains
DIESEL	Gram- Positive	Rods
MICROPLASTIC	Gram- Positive	Mixed culture
MICROPLASTIC	Gram- Positive	Rods
DIESEL + MICROPLASTIC	Gram- Positive	Mixed culture
DIESEL + MICROPLASTIC	Gram -Positive	Mixed culture
DIESEL + MICROPLASTIC	Gram- Positive	Mixed culture
DIESEL + MICROPLASTIC	Gram-Positive	Rods

5.5.1 Negative control:

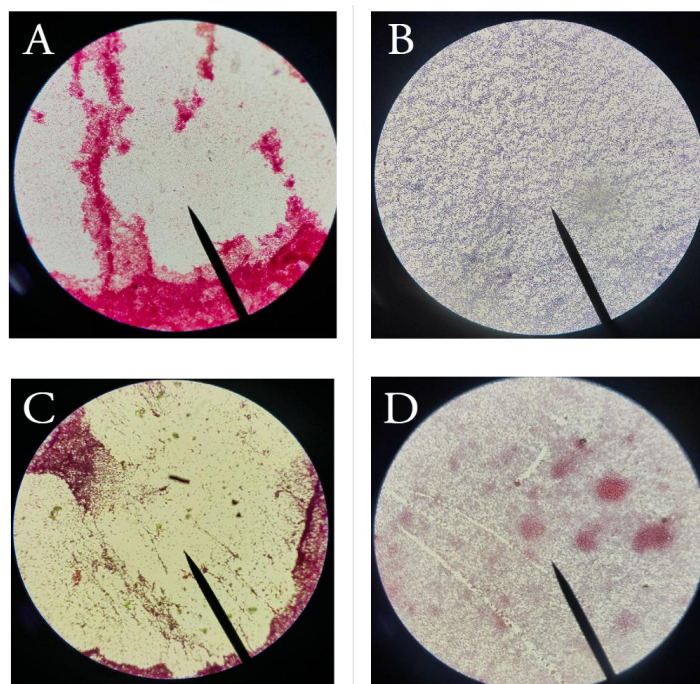


FIG 5.19: A -Control Colony 1; B - Control Colony 2; C - Control Colony 3; D - Control Colony 4

5.5.2 Diesel:

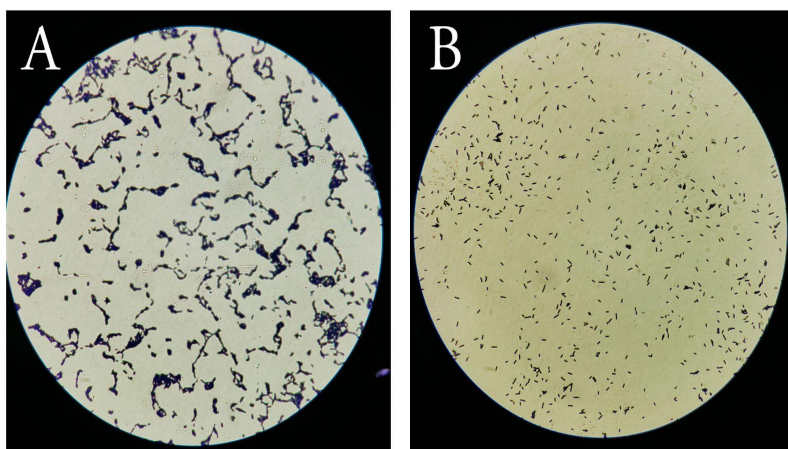


FIG 5.20: A - Diesel Colony 1; B - Diesel Colony 3

5.5.3 Microplastics:

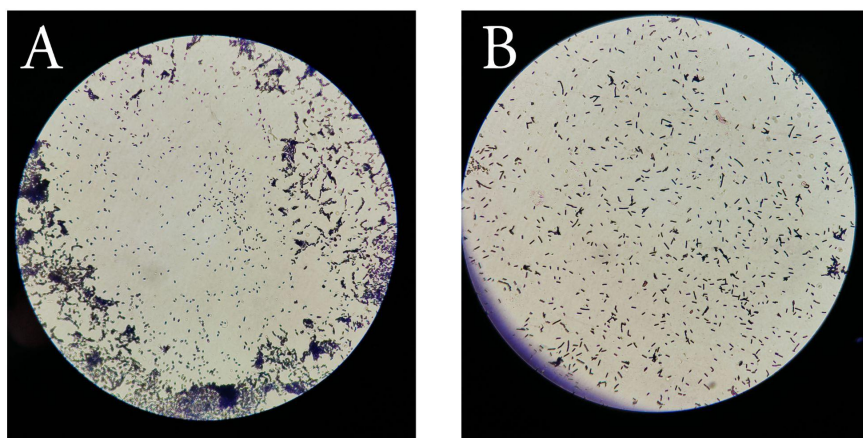


FIG 5.21: A - Microplastics Colony 1; B - Microplastics Colony 2

5.5.4 Diesel + Microplastics:

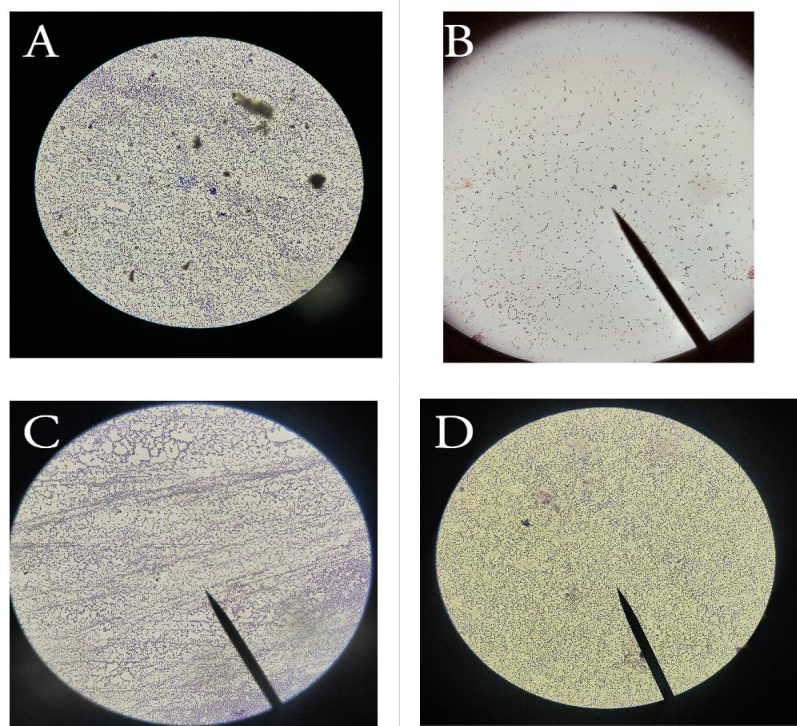


FIG 5.22: A - D+MP Colony 1; B - D+MP Colony 2; C - D+MP Colony 3; D - D+MP Colony 4

5.6 MOTILITY:

The bacteria assessed for motility contained mostly motile bacteria with an exception of two control plate bacteria where the bacteria were non-motile.

**TABLE 5.23 - Motility results of Artemia exposed to different toxicity media
(Negative control, Diesel, Microplastics and Diesel + Microplastics)**

MEDIUM	TYPES
CONTROL (NC)	Motile
CONTROL (NC)	Motile
CONTROL (NC)	Non-motile
CONTROL (NC)	Non-motile
DIESEL	Motile
DIESEL	Motile
MICROPLASTICS	Motile
MICROPLASTICS	Motile
DIESEL + MICROPLASTICS	Motile
DIESEL + MICROPLASTICS	Motile
DIESEL + MICROPLASTICS	Motile
DIESEL + MICROPLASTICS	Motile

5.7 ACUTE GUT EXPOSURE ANALYSIS:

The dyed organism was examined using a Leica Phase contrast microscope after exposure. 10x magnification was used to obtain the images. After 24-hour exposure to the toxicity medium, the organism showed an absence of gut epithelial damage and no gut leakage additionally, no morphological changes were observed in the acute exposure test.

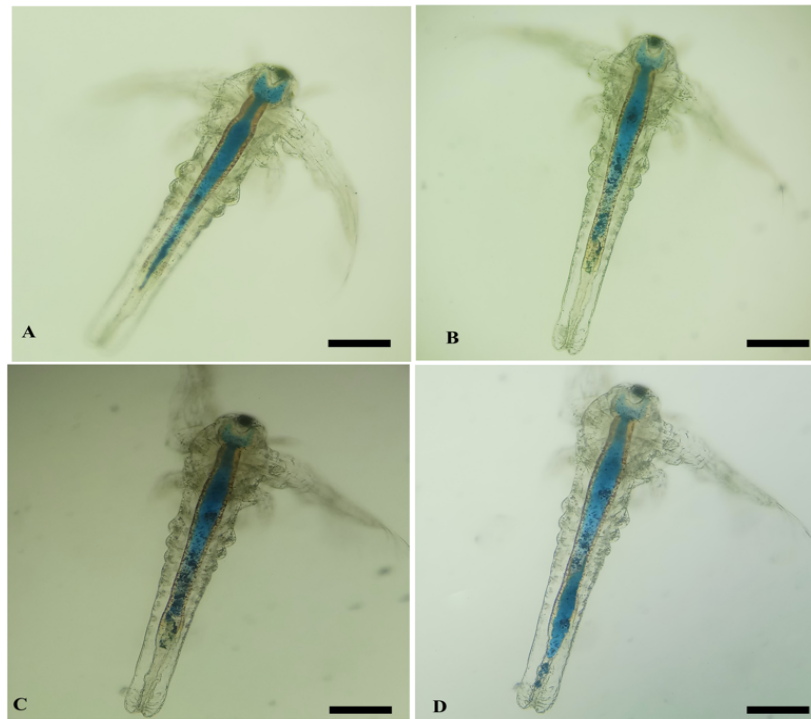


FIG 5.24 A - Negative control; B - Microplastics treated; C - Diesel treated; D - Combined exposure; The dyed organisms were viewed under a phase contrast microscope to evaluate the epithelial damage to the organism and the gut leakage.

DISCUSSION

Microplastics and diesel pollutants are two significant ecological problems that negatively impact aquatic ecosystems. Plastic waste and oil spills in the oceans have resulted in a buildup of these pollutants in marine organisms such as brine shrimp (*Artemia salina*). Although the individual implications of microplastics and diesel pollution on marine organisms have been extensively studied, their combined impacts on brine shrimp remain undetermined. This is of great concern since *Artemia* is a vital source of nourishment for numerous marine organisms and any disruption in their population can have a domino effect on the entire marine food chain. Recognising the consequences of the combined toxicity of microplastics and diesel contamination in brine shrimp is essential in these circumstances for ensuring the long-term viability of marine environments.

The present study is done to assess the toxicity of diesel and microplastics in marine brine shrimp (*Artemia salina*). The level of toxicity that microplastics impart to aquatic organisms, particularly the marine brine shrimp *Artemia*, is assessed using the Lethal Concentration value (LC50) which may vary depending on the conditions of a study (Cole *et al.*, 2013, Lee *et al.*, 2021). According to the results of the LC50 test, the *Artemia* population drops to 50% at 10^5 particles/ml at 48 hours. The toxicity of diesel fuel to marine life has been studied extensively and the LC50 value for diesel might change based on specific conditions. The result obtained from performing the LC50 test compared to the values of 2.2 mg/L (Bonnet *et al.*, 2004) and 3.24 mg/L (Lin *et al.*, 2007) is that the population reduced by 50% in 48 hrs which was less than 5 µl/L when 2007).

The probit analysis was done according to Finney's table and the LC₅₀ value for microplastics is 134896.3 particles/ml and for diesel the LC₅₀ value was calculated to be 38589.002 µl/ml. The Lethal concentration values for the additional microplastic and diesel concentrations were also analysed. The probit analysis was done with reference to the study conducted by Akanksha Singh and Dr Kannez Zahra., 2017.

An extensive species of microbes were found in *Artemia*'s gut which might play an important role in nutrient cycling and other metabolic processes within *Artemia*

(Balcázar *et al.*, 2010). The potential bacteria which were identified by the above preliminary tests include *Vibrio* spp., which belongs to the phylum *Pseudomonadota* (previously known as *Proteobacteria*); *Lactobacillus* spp., and *Ruminococcus* spp., which belongs to the phylum *Firmicutes* and *Flavobacterium* spp., which belongs to *Bacteriodata* were similar to the results *Firmicutes* (37.760- 51.52%), *Bacteriodata* (3.2 - 15.41%) and *Proteobacteria* (33.11-42.01%) which were obtained from the gut of *Artemia* (Yang Xu., 2022). The study conducted by Gabriela Vázquez-Silva *et al.*, 2017 justifies the present study since the findings gathered are similar.`

A. parthenogenetica had been determined to exhibit abnormal intestinal epithelial cell ultrastructures after being subjected to 10-100 particles mL⁻¹ of 10 ml polystyrene microspheres for 24 h. (Wang *et al.* 2019). The intestinal permeability of *A. franciscana* was typically found in the head region. However, in the case of weak damage, the dye penetrates the gut in small quantities until the entire gut content passes through (L. Kim *et al.* 2021). Due to the current study's acute exposure (24 hours), no epithelial or gut damage was observed in *Artemia*.

CHAPTER 6

SUMMARY AND CONCLUSION

The detrimental impacts of microplastics and diesel on marine brine shrimp (*Artemia Salina*) are the subjects of the current research. The present study involves determining the toxicant's lethal concentration (LC₅₀) for the selected model organism. Additionally, it provides a rudimentary understanding of the gut microbiome of brine shrimp and how exposure to diesel, microplastic, or a combination of both might affect it. Furthermore, the brine shrimp's gut damage was evaluated.

Microplastic and diesel pollution, over the past few decades, have been predominant pollutants in the ocean and studies based on both have become a main topic in the research sector. Plastics which are dumped into the sea are ingested by the surface zooplankton. Diesel pollution is major in and near the harbours where the boats used refined diesel to power their boats. These mainly affect the zooplankton present on the ocean's surface. Both of these toxicants affect the physiological and morphological characteristics of the organisms. *Artemia salina*, a zooplankton, is chosen as the model organism for the present study because of its easy adaptability to different conditions and easy hatching of the cysts. Research based on the separate toxicities of diesel and microplastics has been an ongoing topic but the combined effects have not yet been widely studied.

The results of this study revealed that microplastics and diesel had a synergistic effect on brine shrimp mortality which emphasises the need for further investigation into the combined detrimental impact of multiple pollutants in the marine environment. The shrimp nauplii were subjected to different concentrations of microplastics, diesel, and a combination of diesel and microplastic for 24 and 48 hours. The values of the LC₅₀ indicate that the population had decreased by 50% for 1,00,000 particles/ml in microplastics and 1 µl/ml in diesel. Probit analysis was done to obtain the approximate lethal concentration of the test organism exposed to microplastics and diesel at 24 and 48 hours. The results showed that exposure to toxicants were highly susceptible to the test organisms. The demonstration of the microflora encountered in the *Artemia*'s gut resulted in the possible presence of bacteria under the genus *Vibrio* spp., *Lactobacillus*

spp., *Ruminococcus* spp., and *Flavobacterium* spp. which belong under the phyla *Proteobacteria*., *Firmicutes*., and *Bacteroidota*. The gut exposure analysis did not reveal any epithelial damage or gut leakage after 24-hour leakage.

The combined toxicity of diesel and microplastics needs to be studied since it may bring insights into their interactions, synergistic effects, health effects, ecological effects, and management approaches. Such knowledge is crucial for comprehending the intricate interactions between these two common pollutants and the risks they may pose to ecosystems and human health, as well as for guiding decision-making processes aimed at minimising their adverse effects. This study may benefit a wide range of people including environmental and public health agencies, industries and manufacturers, researchers and scientists, the public and communities, and conservation and environmental organisations. The objectives of the following phase of the research might be on figuring out whether the microorganisms already present can break down microplastics and diesel, or the impact of microplastic and diesel stress on the gut microflora of *Artemia* may have negative impacts for humans or other aquatic organisms.

REFERENCE

1. Alimba, C. G., & Faggio, C. (2019). Microplastics in the marine environment: Current trends in environmental pollution and mechanisms of toxicological profile. *Environmental Toxicology and Pharmacology*, 68, 61–74. <https://doi.org/10.1016/j.etap.2019.03.001>
2. Ajith, N., Sundaramanickam, A., Parthasarathy, S., Manupoori, S., & Janakiraman, S. (2020). Global distribution of microplastics and its impact on the marine environment—a review. *Environmental Science and Pollution Research*, 27(21), 25970–25986. <https://doi.org/10.1007/s11356-020-09015-5>
3. Al-Thawadi, S. (2020). Microplastics and Nanoplastics in Aquatic Environments: Challenges and Threats to Aquatic Organisms. *Arabian Journal for Science and Engineering*, 45(6), 4419–4440. <https://doi.org/10.1007/s13369-020-04402-z>
4. Alimba, C. G., & Faggio, C. (2019b). Microplastics in the marine environment: Current trends in environmental pollution and mechanisms of toxicological profile. *Environmental Toxicology and Pharmacology*, 68, 61–74. <https://doi.org/10.1016/j.etap.2019.03.001>
5. Alimba, C. G., & Faggio, C. (2019c). Microplastics in the marine environment: Current trends in environmental pollution and mechanisms of toxicological profile. *Environmental Toxicology and Pharmacology*, 68, 61–74. <https://doi.org/10.1016/j.etap.2019.03.001>
6. Almeda, R., Wambaugh, Z., Wang, Z., Hyatt, C. J., Liu, Z., & Buskey, E. J. (2013). Interactions between zooplankton and crude oil: toxic effects and bioaccumulation of polycyclic aromatic hydrocarbons. *DOAJ* (DOAJ: Directory of Open Access Journals). <https://doi.org/10.1371/journal.pone.0067212>

7. Biodiversity and World Bank. (n.d.). World Bank.
<https://www.worldbank.org/en/topic/biodiversity>
8. Barboza, L. G. A., Cózar, A., Gimenez, B. C., Barros, T. L., Kershaw, P., & Guilhermino, L. (2019). Macroplastics Pollution in the Marine Environment. Elsevier eBooks, 305–328. <https://doi.org/10.1016/b978-0-12-805052-1.00019-x>
9. Balcázar, J. L., Lee, N., Pintado, J., & Planas, M. (2010). Phylogenetic characterization and in situ detection of bacterial communities associated with seahorses (*Hippocampus guttulatus*) in captivity. *Systematic and Applied Microbiology*, 33(2), 71–77. <https://doi.org/10.1016/j.syapm.2009.11.005>.
10. Cerasa, M., Teodori, S., & Pietrelli, L. (2021). Searching Nanoplastics: From Sampling to Sample Processing. *Polymers*, 13(21), 3658. <https://doi.org/10.3390/polym13213658>
11. Cole, M. T., Lindeque, P., Halsband, C., & Galloway, T. S. (2011). Microplastics as contaminants in the marine environment: A review. *Marine Pollution Bulletin*, 62(12), 2588–2597. <https://doi.org/10.1016/j.marpolbul.2011.09.025>
12. Chen, J., & Denison, M. S. (2011). The Deepwater Horizon oil spill: the environmental fate of the oil and the toxicological effects on marine. . . ResearchGate.
https://www.researchgate.net/publication/228411392_The_Deepwater_Horizon_oil_spill_environmental_fate_of_the_oil_and_the_toxicological_effects_on_marine_organisms
13. Cole, M. T., Lindeque, P., Fileman, E. S., Halsband, C., Goodhead, R. M., Moger, J., & Galloway, T. S. (2013). Microplastic Ingestion by Zooplankton. *Environmental Science & Technology*, 47(12), 6646–6655. <https://doi.org/10.1021/es400663f>

14. Dris, R., Gasperi, J., Mirande, C., Mandin, C., Guerrouache, M., Langlois, V., & Tassin, B. (2017). A first overview of textile fibres, including microplastics, in indoor and outdoor environments. *Environmental Pollution*, 221, 453–458. <https://doi.org/10.1016/j.envpol.2016.12.013>
15. Dhont, J., Dierckens, K., Støttrup, J., Van Stappen, G., Wille, M., & Sorgeloos, P. (2013). Rotifers, Artemia and copepods as live feeds for fish larvae in aquaculture. Elsevier eBooks, 157–202. <https://doi.org/10.1533/9780857097460.1.157>
16. Eriksen, M., Lebreton, L., Carson, H. J., Thiel, M., Moore, C. E., Borerro, J. C., Galgani, F., Ryan, P. G., & Reisser, J. (2014). Plastic Pollution in the World's Oceans: More than 5 Trillion Plastic Pieces Weighing over 250,000 Tons Afloat at Sea. *PLOS ONE*, 9(12), e111913. <https://doi.org/10.1371/journal.pone.0111913>
17. Edward, J. P., Mathews, G., Raj, K. D., Laju, R. L., Bharath, M. S., Kumar, P. S., Arasamuthu, A., & Grimsditch, G. (2020). Marine debris — An emerging threat to the reef areas of the Gulf of Mannar, India. *Marine Pollution Bulletin*, 151, 110793. <https://doi.org/10.1016/j.marpolbul.2019.110793>
18. Eom, H., Nam, S., & Rhee, J. (2020). Polystyrene microplastics induce mortality through acute cell stress and inhibition of cholinergic activity in a brine shrimp. *Molecular & Cellular Toxicology*, 16(3), 233–243. <https://doi.org/10.1007/s13273-020-00088-4>
19. Freinkel, S. (2011, May 29). A Brief History of Plastic's Conquest of the World. *Scientific American*. <https://www.scientificamerican.com/article/a-brief-history-of-plastic-world-conquest/>

20. FAO Fisheries & Aquaculture. (n.d.).
<https://www.fao.org/fishery/en/openasfa/e41a30ad-0176-4471-b1f2-792692ac2a99>
21. Geyer, R. (2020). A Brief History of Plastics. Springer eBooks, 31–47.
https://doi.org/10.1007/978-3-030-38945-1_2
22. Geyer, R. (2020b). Production, use, and the fate of synthetic polymers. Elsevier eBooks, 13–32. <https://doi.org/10.1016/b978-0-12-817880-5.00002-5>
23. Hale, R. C., Seeley, M., La Guardia, M. J., Mai, L., & Zeng, E. Y. (2020). A Global Perspective on Microplastics. Journal of Geophysical Research: Oceans, 125(1).
<https://doi.org/10.1029/2018jc014719>
24. Han, Y., Nambi, I. M., & Clement, T. P. (2018). Environmental impacts of the Chennai oil spill accident – A case study. Science of the Total Environment, 626, 795–806. <https://doi.org/10.1016/j.scitotenv.2018.01.128>
25. Honda, M., & Suzuki, N. (2020). Toxicities of Polycyclic Aromatic Hydrocarbons for Aquatic Animals. International Journal of Environmental Research and Public Health, 17(4), 1363. <https://doi.org/10.3390/ijerph17041363>
26. ITOPF.(n.d.).Statistics.
<https://www.itopf.org/knowledge-resources/data-statistics/statistics/>
27. Kingston, P. (2002). Long-term Environmental Impact of Oil Spills. Spill Science & Technology Bulletin, 7(1–2), 53–61.
[https://doi.org/10.1016/s1353-2561\(02\)00051-8](https://doi.org/10.1016/s1353-2561(02)00051-8)
28. Koru, E. (2023). Recent Developments On The Application Of Artemia In The Ornamental Fish Culture. ResearchGate.

https://www.researchgate.net/publication/267704641_Recent_Developments_On_The_Application_Of_Artemia_In_The_Ornamental_Fish_Culture

29. Kim, L., Kim, S., Kim, T. Y., Kim, J., & An, Y. (2021). Synthetic and natural microfibers induce gut damage in the brine shrimp *Artemia franciscana*. *Aquatic Toxicology*, 232, 105748. <https://doi.org/10.1016/j.aquatox.2021.105748>
30. Lee, Y., Yoon, D., Lee, Y. H., Kwak, J. Y., An, Y., Lee, J., & Park, J. Y. (2021). Combined exposure to microplastics and zinc produces sex-specific responses in the water flea *Daphnia magna*. *Journal of Hazardous Materials*, 420, 126652. <https://doi.org/10.1016/j.jhazmat.2021.126652>
31. Lechthaler, S., Waldschläger, K., Stauch, G., & Schüttrumpf, H. (2020). The Way of Macroplastic through the Environment. *Environments*, 7(10), 73. <https://doi.org/10.3390/environments7100073>
32. Marine pollution control and Management by Dr Tanmoy Rudra
33. Mattsson, K., Jovic, S., Doverbratt, I., & Hansson, L. (2018). *Nanoplastics in the Aquatic Environment*. Elsevier eBooks, 379–399. <https://doi.org/10.1016/b978-0-12-813747-5.00013-8>
34. Mei, H., & Yin, Y. (2009). Studies on marine oil spills and their ecological damage. *Journal of Ocean University of China*, 8(3), 312–316. <https://doi.org/10.1007/s11802-009-0312-5>
35. Martínez-Álvarez, I., Menach, K. L., Devier, M., Cajaraville, M. P., Budzinski, H., & Orbea, A. (2022). Screening of the Toxicity of Polystyrene Nano- and Microplastics Alone and in Combination with Benzo(a)pyrene in Brine Shrimp Larvae and Zebrafish Embryos. *Nanomaterials*, 12(6), 941. <https://doi.org/10.3390/nano120609>

36. Plastics use in 2019. (n.d.). OECD Environment Statistics | OECD iLibrary. https://www.oecd-ilibrary.org/environment/data/global-plastic-outlook/plastics-use-in-2019_eff24eb-en
37. Plastics Europe. (2023, March 24). Plastics Europe • Enabling a sustainable future. <https://plasticseurope.org/>
38. Ritchie, H. (2022, December 19). Biodiversity. Our World in Data. <https://ourworldindata.org/biodiversity-and-wildlife#:~:text=Despite%20dominating%20the%20planet%20in,lives%20in%20the%20marine%20environment>.
39. Ritchie, H. (2018, September 1). Plastic Pollution. Our World in Data. <https://ourworldindata.org/plastic-pollution>
40. Roser, M. (2022, May 4). Oil Spills Our World in Data. <https://ourworldindata.org/oil-spills>
41. Salimi, P., Creed, J. C., Esch, M. M., Fenner, D., Jaafar, Z., Levesque, J. C., Montgomery, A., Salimi, M. A., Edward, J. P., Raj, K. D., & Sweet, M. (2021). A review of the diversity and impact of invasive non-native species in tropical marine ecosystems. *Marine Biodiversity Records*, 14(1). <https://doi.org/10.1186/s41200-021-00206-8>
42. Saadoun, I. (2015). Impact of Oil Spills on Marine Life. InTech eBooks. <https://doi.org/10.5772/60455>
43. Sukumaran, S., Mulik, J., Rokade, M., & Kamble, A. S. (2014). Impact of 'Chitra' Oil Spill on Tidal Pool Macrobenthic Communities of a Tropical Rocky Shore (Mumbai, India). *Estuaries and Coasts*, 37(6), 1415–1431. <https://doi.org/10.1007/s12237-014-9791-8>

44. Suman, T., Jia, P., Li, W., Junaid, M., Xin, G., Wang, Y., & Pei, D. (2020). Acute and chronic effects of polystyrene microplastics on brine shrimp: First evidence highlighting the molecular mechanism through transcriptome analysis. *Journal of Hazardous Materials*, 400, 123220. <https://doi.org/10.1016/j.jhazmat.2020.123220>
45. The Age of Plastic: From Parkesine to Pollution | Science Museum. (n.d.). Science Museum. <https://www.sciencemuseum.org.uk/objects-and-stories/chemistry/age-plastic-parkesine-pollution#:~:text=While%20we%20think%20of%20plastic.products%2C%20from%20medallions%20to%20cutlery>
46. Thushari, G., & Senevirathna, J. (2020). Plastic pollution in the marine environment. *Heliyon*, 6(8), e04709. <https://doi.org/10.1016/j.heliyon.2020.e04709>
47. Teuten, E. L., Rowland, S. J., Galloway, T. S., & Thompson, R. F. (2007). Potential for Plastics to Transport Hydrophobic Contaminants. *Environmental Science & Technology*, 41(22), 7759–7764. <https://doi.org/10.1021/es071737s>
48. Vázquez-Silva, G., Ramírez-Saad, H., Aguirre-Garrido, J. F., Mayorga-Reyes, L., Azaola-Espinosa, A., & Morales-Jiménez, J. (2017). Effect of bacterial probiotics bio encapsulated into *Artemia franciscana* on weight and length of the shortfin silverside (*Chirostoma humboldtianum*), and PCR DGGE characterization of its intestinal bacterial community. *Latin American Journal of Aquatic Research*, 45(5), 1031–1043. <https://doi.org/10.3856/vol45-issue5-fulltext-18>
49. Wang, Z., Fingas, M., & Page, D. S. (1999). Oil spill identification. *Journal of Chromatography A*, 843(1–2), 369–411. [https://doi.org/10.1016/s0021-9673\(99\)00120-x](https://doi.org/10.1016/s0021-9673(99)00120-x)

50. Xu, Y., Cheng, H., Meng, J., Xu, B., Li, X., Peijing, S., Huang, Z., & Gao, Q. (2023). Study on the quality and symbiotic microbial composition of *Artemia* nauplii in three main producing areas. *Journal of Freshwater Ecology*, 38(1), 1–12. <https://doi.org/10.1080/02705060.2022.2158140>