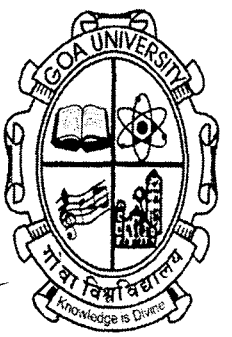
GENOTOXICITY STUDIES IN SELECTED FINFISH AND SHELLFISH ALONG THE GOAN COAST

A thesis submitted to Goa University for the award of the Degree of

DOCTOR OF PHILOSOPHY

In

ZOOLOGY



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March, 2018

Dedicated to my Parents

CERTIFICATE

This is to certify that the thesis entitled "GENOTOXICITY STUDIES IN SELECTED FINFISH AND SHELLFISH ALONG THE GOAN COAST" submitted by Mr. Avelyno Hubert D'Costa, for the award of the degree of Doctor of Philosophy in Zoology, Goa University, is based on the original studies carried out by him under my supervision. This thesis or any part thereof has not been previously submitted for any other degree/diploma in any other universities or institutions in India or abroad.

Place:

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DECLARATION

I, Avelyno Hubert D'Costa, hereby declare that the present thesis entitled "GENOTOXICITY STUDIES IN SELECTED FINFISH AND SHELLFISH ALONG THE GOAN COAST" is my original contribution and the same work has not submitted to any other universities or tertiary institutions in India and abroad for any degree/diploma. The literature related to the problem investigated has been cited. The work was undertaken at the Department of Zoology, Faculty of Life Sciences and Environment, Goa University.

Place: GU Campus

Date: 26/11/18

Avelyno Hubert D'Costa

(Research Scholar)

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March, 2018

Abbreviations

%	Percentage
AChE	Acetylcholinesterase
CAT	Catalase
CI	Condition Index
Cd	Cadmium
Cu	Copper
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DW	Distilled water
EDTA	Ethylenediaminetetraacetic acid
Fe	Iron
gm	gram
h	Hours
>	Greater than
<	Less than
L	Litre
ml	Millilitre
µL	Microlitre
µg	Microgram
M	Monsoons
mA	Milliampere
MDA	Malondialdehyde
MN	Micronucleus
Mn	Manganese
Pb	Lead
SD	Standard deviation
TPH	Total petroleum hydrocarbon
V	Volts

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Background / General Introduction

Genetic toxicology is a branch of toxicology that deals with the studies on the genotoxic effects of substances, including the induction of mutation by chemical means. It examines the interaction of chemical and physical agents with genetic material, in relation to subsequent adverse effects, such as alterations in somatic cells (cancer) or alterations in germ cells (genetic disease in future generations). In other words, genetic toxicology is a branch of toxicology that identifies and analyses the action of agents with toxicity directed towards the hereditary components of living systems. It can be widely implemented for identifying the genotoxic agents found in the environment, whose presence may alter the integrity of the gene pool of wide range of organisms including human beings. It can also be used for the detection and mechanistic understanding of the action of carcinogenic agents.

The aquatic environment plays a vital role in the functioning of our ecosystem and is intimately related with human health. With increasing demands for industrial development, the problem of disposal of anthropogenic chemicals, wastes and by-products in the aquatic environment has become a major cause for concern. A majority of these chemical contaminants contain potential genotoxic and / or carcinogenic substances. These substances, called genotoxins, can produce alterations in nucleic acids at sub-toxic exposure levels, resulting in the modified DNA sequences such as point mutations or DNA inactivation. The DNA damage induced as a result of this exposure can cause a variety of effects such as mutagenesis, clastogenesis, malignancies, altered protein expression, reduced fertility, reduced growth, abnormal development and reduced survival of organisms. Further, genotoxicity not only reduces the 'fitness' (i.e. growth, fertility and fecundity) in wild aquatic populations, but may also pose a risk to human health via the food chain.

To understand the biological state or condition of the organism, a measurable indicator should be used which is referred to as a biomarker. Biomarkers used in aquatic environments are relatively simple tests that are thus employed to identify and evaluate contaminants, their toxicity and bioavailability to organisms. These biomarkers serve as early warnings for the presence of toxic xenobiotics, which then make it possible to implement corrective measures before organisms, their communities or even the ecosystem suffers irreversible damage.

Marine and estuarine pollution includes a range of threats including from land-based sources, oil spills, untreated sewage, heavy siltation, eutrophication (nutrient enrichment), invasive species, persistent organic pollutants (POP's), heavy metals from mine tailings and other sources, acidification, radioactive substances, marine litter and the destruction of coastal and marine habitats by overfishing. Many of these pollutants accumulate at the depths, where they are consumed by marine organisms and introduced into the global food chain.

Goa, located on the west coast of India, faces the Arabian sea and is situated between the states of Maharashtra in the north and Karnataka in the south. Being the smallest state in India in terms of size, it has a coastline length of 105 km and lies in an area known as Konkan belt of India. Besides the open access to the coast on the western side, Goa is also bound by the prominent hills of the Western Ghats on the east called the Sahyadris. This being the catchment area of the monsoons which occur during the months from June to September in Goa is covered with dense forests. This plant cover helps in the percolation of rainwater and increase the water table in Goa by preventing the runoff of rainwater. Runoff of rainwater is not only responsible for the decreased water table, but also results in the decreasing fertility of the land by taking fertile black soil of the land surface with it. Further, forests conserve the rain water and are prime sources of the major Goan rivers, the lifeline of

the land. The coastal region can be divided into two interrelated ecozones – the coastal region proper and the flood plains / alluvial flats. These latter ones are formed by the rivers by the deposit of eroded material from the Sahyadris along their banks. These are areas of rich cultivation with rural settlements of agricultural and fishing populations. All coastal fisheries on the west coast depend on the nutrient discharge into the coastal seas and the subsequent marine productivity. A majority of the iron and ferromanganese ores that are mined in Goa are transported through the riverine systems in barges. In addition these rivers provide a cheap mode of transportation to the people, apart from being the only source of fish during monsoon when fishing activities in the open sea are suspended due to their breeding season and also due to rough weather. The Mandovi and Zuari estuaries are the main waterways of Goa and are of utmost importance for the economy of the territory as they flow through mining areas opening into the Arabian Sea. The two river mouths are separated by a promontory between them at Cabo. Developmental activities are more along these two river basins as compared to the rest of the territory. A number of anthropogenic activities take place along the coasts, rivers and backwaters in Goa including infrastructure and building construction, land reclamation, barge building yards, beach shacks, water sports, river cruises, casinos, sewage disposal, farming and pisciculture and ore transport by barges.

The state has the most extensive mining ratio in the country. Ongoing operation of more than 800 mines, many of which are located just few meters away from the river banks, are causing severe pollution by their mining rejects. Every year the heavy monsoon run off carries the overflow and washes off from the huge quantum of mining rejects into the rivers. These mining rejects along with slime and pumped out turbid water from deep mining pits are then transported further downstream by the rivers. As a result, the Mandovi and Zuari are also

heavily polluted with mining rejects. While Zuari has 10 operating mines in its catchment, the Mandovi has 27 mines depositing 70,000 cubic tonnes of iron particulates every year in the Mandovi River. As the iron ore is transported by barges through the rivers to the Mormugao harbor at Vasco, oil and grease along with mineral ore, pollute adversely these rivers. Ammonium Nitrate used as the explosive for mining, adds nitrates to the river water causing eutrophication. Washing and cleaning of mining trucks done in the rivers and streams also intensifies water pollution. All these factors introduce suspended as well as dissolved particles, petroleum hydrocarbons, industrial effluents and domestic sewage into the estuaries, creating additional stress to the ecosystem.

Goa's coastline also lies off the oil tanker route from the Gulf ports to the Far east and Japan. Every year, millions of tonnes of oil and its products are transported along this route. Ships sailing along this route discharge their oily ballasts and bilge washings. After evaporations of oil and photooxidation, the heavier fractions gradually form into tar balls. Driven by winds and currents, these tar balls are deposited on the beaches. Periodic tar balls and raw oil pollution is observed on all the major beaches in Goa, mainly during the onset of the monsoon season (tar balls) and sometimes throughout the year (raw oil cakes). The consequence of such pollution in the rivers eventually results in the deterioration of health and ultimately loss of riverine and estuarine fauna. Further, the potential of estuarine and marine fauna to accumulate such pollutants may enhance the adverse effects of these compounds on their genome and induce drastic changes in their physiological and biochemical characters. Seafood is a major source of income for the coastal state of Goa and is the staple diet of the locals. Hence, effects in the aquatic fauna could directly or indirectly affect humans as well.

The present study therefore aims to evaluate the genotoxicity in finfish and shellfish inhabiting areas of anthropogenic stress along the coast of Goa. Although a number of reports are available on the genotoxicity studies in various finfish and shellfish, studies along the Goan coast are limited. Additionally, very limited literature is available on the genotoxicity of heavy metals and total petroleum hydrocarbons from Goa. To fill in this lacuna of information, the following objectives were formulated.

Objectives of the study

- To know the extent of genetic damage present (prevailing) in the marine organisms such as finfish and shellfish along the Goan coast and to identify the probable mutagenic pollutants from the tissues.
- To identify the mutagenic pollutants present along the Goan coasts.
- To confirm their genotoxicity using laboratory tests on experimental animals.
- To develop a protocol to use biomarkers to provide early warnings of deleterious / genotoxic effects of pollution on the marine ecosystem.

Based on these objectives, the research work in this thesis is presented in four chapters, as follows:

Chapter 1: Heavy metals and petroleum hydrocarbons as pollutants in the environment

Chapter 2: Mutagenic pollutants and genetic damage in the fauna of the Goan coast

Chapter 3: Laboratory confirmation of genotoxic potential of the pollutants

Chapter 4: Biochemical and physiological tests as biomarkers of genotoxicity

Chapter 1:

Heavy metals and petroleum hydrocarbons as pollutants in the environment

1.1. Introduction

Marine pollution includes a wide range of threats of diverse origin including land-based sources, oil spills, untreated sewage, heavy siltation, eutrophication (nutrient enrichment), invasive species, persistent organic pollutants (POP's), heavy metals from mine tailings and other sources, acidification, radioactive substances, marine litter, overfishing and destruction of coastal and marine habitats. Many of these pollutants accumulate in the bottom waters and sediment, where they are consumed by marine organisms and introduced into the global food chain.

Heavy metals occur naturally in the aquatic environment mostly at very low concentrations. The increased concentrations of these pollutants is attributed to various anthropogenic activities such as mining, offshore oil exploration, discharge of industrial effluents such as pesticides, fertilizers, medical waste as well as agricultural runoffs. The toxicity of a metal depends on its nature and concentration in the environment. Some of the metals are essential for the survival of aquatic organisms at specific concentrations. Essential metals may also be toxic at very high concentrations whereas non-essential metals may not be toxic at low concentrations. The solubility of these metals in water depends on various factors such as pH, salinity, temperature and nature of other associated ions. Metals may also interact with suspended particles in the water and this may be one of the mechanisms of their concentration and distribution patterns. These metals ultimately get incorporated in the aquatic sediments, the concentrations of which usually exceed those in the water column by several folds of magnitude. These high concentrations can pose several implications to organisms inhabiting the bottom niche and benthic feeders.

Discharge of heavy metals into river or any marine environment can induce drastic changes in marine species diversity as well as the marine ecosystem, due to their toxicity and accumulative behavior (Bakan and Böke Özkoç, 2007; Bat *et al.*, 2009). Marine organisms such as fish can accumulate heavy metals to concentrations many times higher than present in water or sediment (Bat *et al.*, 2009; Boran and Altinok, 2010)

Petroleum refers to any hydrocarbon mixture that may be present as gases, liquids and condensates. The liquid phase is commonly referred to as crude oil. This portion is converted into commercial form by distillation which removes impurities and enables production of various fractions such as gasoline, kerosene and oil. Petroleum is the major source of energy powering the world economy. Most of this petroleum is used to generate energy primarily for transportation. However, it is also very important in the manufacture of petrochemicals used in the synthesis of other products such as synthetic rubber, plastics and detergents. There are several sources of petroleum hydrocarbon pollution. However, human activities such as oil transportation and spills, shipping, industrial, storm-water and domestic discharges are believed to have important influence on the presence of these compounds in the marine environment. Petroleum hydrocarbons, especially the polycyclic aromatic hydrocarbons (PAHs), are a widespread group of pollutants which may enter the environment by various processes such as incomplete combustion of organic matter of pyrolytic origin at higher temperatures, slow maturation of organic matter of petrogenic origin under geochemical gradient conditions and degradation of petroleum products and precursors. Most inputs of petroleum hydrocarbons are due to anthropogenic activity and are considered to be the major source of these compounds in the environment such as wastes from urbanized and

industrialized areas, offshore petroleum hydrocarbon production or petroleum transportation. Further, PAHs are highly toxic to marine organisms even at very low concentrations.

1.1.1. Metal and petroleum hydrocarbon pollution in Goan coastal waters

Contamination of water and sediment by organic and inorganic pollutants along the Goan coast is of a major concern. According to the Indian Bureau of Mines of the Ministry of Mines (Government of India), Goa accounts for 18% of the iron ore and 3% of the manganese ore in India (IBM, 2014 a; b). Metal effluents enter into the Goan estuaries during ferro-manganese mining activities and may pose a threat to the fauna and flora inhabiting these waters (Attri and Kerkar, 2011). These metals are then carried downstream by the effect of water currents in the estuarine regions (Alagarsamy, 2006) and may either circulate in the water column or may get deposited on the surface sediment and interact with the associated benthic fauna. Additionally, various by-products of the mining processes, which may be potential carcinogens, also find their way into the water due to the lack of proper disposal systems and contaminate the surrounding waters (Koski, 2012). According to Dessai and Nayak (2008), the surface sediments in the Zuari estuary are contaminated mainly by manganese, followed by cobalt and least with chromium, zince, copper and iron. They further reported that the major source of these metals is mining and its associated activities such as ore transportation to offshore platforms, ore loading and barge building. The Zuari estuary receives considerable amount of petroleum hydrocarbons due to the discharge of waste materials from the Mormugao harbour and an agrochemical industry (Sarkar *et al.*, 2014). The occurrence of tar balls, which is a common phenomenon along the Goa coast during the monsoon season, may affect water quality and may also induce carcinogenicity in the marine fauna (Suneel *et al.*, 2013).

A majority of these pollutants are toxic to marine organisms and also to other organisms in the food chain. Exposure to these can lead to abnormal physiological responses and can adversely affect the growth, development, reproduction and behaviour of aquatic organisms (Bistodeau *et al.*, 2006; Ginebreda *et al.*, 2014) and can induce teratogenesis, clastogenesis and oncogenesis in them as well (Gangar *et al.*, 2010; Yang *et al.*, 2010). The risk of human cancer is greatly increased due to their exposure to genotoxic contaminants such as polycyclic aromatic hydrocarbons (PAHs) and heavy metals through the food chain.

1.2. Review of Literature

A number of reports are available on the presence of heavy metals in the estuarine habitat as a result of human activities. The sediment of the Pearl river estuary in China is reported to be contaminated with heavy metals such as Cd, Zn and Ni due to various developmental activities occurring near this area (Li *et al.*, 2007). Ruzhong *et al.* (2010) observed significant Cu and Cd in the surface sediment of another estuary of the Tangxi River in China. In the Tamaki estuary in New Zealand, the sediment was found to be significantly contaminated with heavy metals such as Cu, Pb, Zn and Cd as a result of urbanization compared to baseline values obtained from a pristine location (Abrahim and Parker, 2008). According to a report by Kehrig *et al* (2003), the Guanabara Bay in Brazil receives inputs of untreated industrial wastes which comprise of oil, heavy metals and organic matter from the industrial activities in Rio de Janeiro. They also observed significant concentrations of Cd, Pb and Cu in the surface sediments which could potentially affect the mangrove vegetation and the associated fauna of this region. Heavy metals present in the water and sediments often get bioaccumulated in the tissues of organisms inhabiting these areas. Vicente-Martorell *et al.* (2009) reported high concentrations of Cu, Zn, Pb, Cd and As in the water and sediment of a

polluted estuary in Spain. The concentrations of these metals were also found to be elevated in the tissues of two fish species *Sparus aurata* and *Solea senegalensis* inhabiting this estuary indicating the presence of these metals in the estuarine environment. A number of reports are available on the quantum of heavy metals prevailing along the Indian coast. Kumar and Patterson (2009) reported that the sediment of the Manakudy estuary near the southern tip of India in Tamil Nadu is moderately contaminated with metals such as Cr, Ni, Cu, Co, Pb, Zn and Cd and were found to be associated with Fe and Mn ions. Heavy metals such as Cd, Pb, Co and Cu were found to be enriched in the sediment of the Sudarbans mangrove region as well as the Hooghly estuary in north-east India (Banerjee *et al.*, 2012). The reason for this enrichment was attributed to anthropogenic sources such as wastewater effluents from industries and shipping activities rather than from natural sources. Sundaray *et al.* (2011) observed considerable metal contamination in the sediment of Mahanadi basin and reported that that the bioavailability of heavy metals such as Cd, Ni, Co and Pb in the estuarine sediments may pose a threat to the biota inhabiting these waters.

The marine and estuarine environments are also reported to be polluted with diverse petroleum hydrocarbons from various anthropogenic sources. Readman *et al.* (2002) reported that the surface sediments of the Black Sea are contaminated with high concentrations of petroleum hydrocarbons primarily due to discharge from rivers, navigation routes and ports. Significantly high concentrations of petroleum hydrocarbons were also reported in the coastal zones of France which were attributed to discharge of petroleum products and fuel combustion emissions from docked ships (Soclo *et al.*, 2000). The presence of petroleum hydrocarbons in the aquatic environment may also be due to terrestrial sources such as dust. In a study conducted by Boonyatumanond *et al.* (2006), the petroleum hydrocarbons in the

coastal and riverine water of Thailand were analyzed and found to have the same chemical signature as the petroleum hydrocarbons in street dust. Surface sediments from the Yangtze river estuary in China were found to be contaminated with polycyclic aromatic hydrocarbons due to the discharge of wastewater effluents from major cities such as Shanghai (Yamei *et al.*, 2009). In India, Chouksey *et al.* (2004) reported significant petroleum hydrocarbon residues in the bottom water as well as in the surface sediment of Bassien-Mumbai compared to less polluted areas of Ratnagiri and Dabhol. The high concentration of petroleum hydrocarbons is attributed to the release of effluents from industries such as refineries and petrochemical complexes, transport of crude oil and its products at the Mumbai harbour. More recently, Rao *et al.* (2016) also observed significant concentrations of total petroleum hydrocarbons (TPHs) in the water of the Amba estuary which opens into the Mumbai harbor. In Chennai, the surface sediments of Kuvam and Adyar estuaries were reported to be contaminated primarily with petroleum hydrocarbons from the Chennai port (Venkatachalapathy *et al.*, 2010). In the Mandovi estuary in Goa, Veerasingham *et al.* (2015) reported a broad range of petroleum hydrocarbons in the surface sediment and was found to be due to discharge of land based petroleum products and effluents, emission of fly ash from vehicles and industries, and combustion of petrol from barges and ships.

A few reports are available on the pollution of coastal water by heavy metals and petroleum hydrocarbons. Muniz *et al.* (2004) reported heavy metal and petroleum hydrocarbon contamination of the sediments of Montevideo harbor in Uruguay. The presence of heavy metals was due to the release of untreated wastewaters from tanneries whereas that of petroleum hydrocarbons was due to discharges from a thermoelectric centre and a major petroleum refinery. Heavy metal and PAH pollution was also observed in the surface

sediment of the Naples harbor in Italy which is an area of commercial significance (Sprovieri *et al.*, 2007). These pollutants are due to diverse kinds of anthropogenic activities such as industrial and port activities such as shipbuilding, commercial effluent and urban wastewater discharges. Yang *et al.* (2015) estimated the concentrations of heavy metals and petroleum hydrocarbons in the sediments of the Shuangtaizi estuary in China and further reported that Cd, Hg and petroleum hydrocarbons posed an ecological risk to this estuarine environment. Reddy *et al.* (2005) reported seasonal variations in the concentrations of heavy metals and total petroleum hydrocarbons in the Gulf of Cambay in Gujarat, India. This region suffers considerable pollution due to the presence of Alang-Sosiya scrapping yard, which is one of the largest ship-breaking yards in the world.

1.3. Materials and Methods

1.3.1. Study sites

Vasco, Miramar and Dona Paula were selected as the probable polluted sites and Palolem was selected as an unpolluted reference site (Plate 1.1), based on the reports of Sarkar *et al.* (2008, 2010) and Sarker and Sarkar (2015). Palolem is a pristine beach with no known industrial activity at this site. Vasco, an industrial hub, is dependent on the Mormugao harbour for most of its economic activities. This harbour serves as a port for carrier barges, ore barges, oil liners and cruise liners. Miramar is a popular beach located at the mouth of the Mandovi river which is one of the main channels for the transport of iron and manganese ore from upstream sites. Dona Paula is located at the confluence of the Mandovi and Zuari river the latter being used to transport ores. Additionally, effluents released from some factories which are situated on the banks of the river may be carried downstream. Thus, the water, sediment and even the fauna in these areas may suffer considerable anthropogenic stress.

1.3.2. Sampling seasons

Sampling was carried out seasonally for a duration of two years. Based on the occurrence of the monsoons, there are three seasons in Goa: pre-monsoon season from February to May, Monsoon season from June to September and Post-monsoon season from October to January. In the present study, sampling commenced from the monsoon season of 2012 and ended by the end of the pre-monsoon season of 2014. Samplings were done twice per season for a better interpretation of statistical analysis.

1.3.3. Water and sediment collection

Surface and bottom water (2 L each) were collected with the help of a Niskin Sampler and used for various analyses. The temperature (in degrees Celsius: °C), pH and salinity (in ppt) of the water samples were immediately recorded with the help of an automated water and sediment analysis kit (Labtronics, LT-59) consisting of probes for measuring the required parameter. Water samples for dissolved oxygen (DO) were taken carefully without introducing bubbles in amber-coloured bottles (300 mL) and fixed with 1 ml of Winkler's A solution and 1 ml of Winklers B solution. The bottles were stoppered and inverted a few times to allow the formation of a precipitate. The samples for the analysis of phosphates, nitrates, trace metals and TPHs were stored in clean and labeled polypropylene bottles. Sediment samples (~ 500 g) were collected with the help of a Van-Veen Grab and stored in clean and labelled polythene bags. These samples were stored at 4°C and carried to the laboratory for further analysis. Samples were collected from three sampling stations in triplicates at each site during the pre-monsoon, monsoon and post-monsoon periods from March 2012 to February 2014.

1.3.4. Quality assurance and quality control

The appropriate quality assurance methods of sample preparation, handling and preservation were carried out in accordance with US EPA procedures. Bombay High crude oil was the certified reference material for TPH estimation and was also used to prepare the calibration curve. "TraceCERT certified reference metals for AAS (Sigma)" were used for trace metal estimation.

1.3.5. Physico-chemical parameters

The physico-chemical parameters such as temperature, pH, salinity and dissolved oxygen were analysed and recorded as mentioned earlier. Dissolved oxygen content of water samples fixed with Winkler's solutions were estimated by dissolving the precipitate with 1 ml of concentrated sulphuric acid, stoppered and mixed by inverting. 100 ml of this solution was then taken in a conical flask and titrated against sodium thiosulphate till a straw colour developed. Then 1 ml of starch solution was added to obtain a blue colour in the solution. This was then further titrated till the solution became colourless. The concentration of the DO in the sample is equivalent to the ml of titrant used and the values were expressed as mg/L.

Nitrates were analyzed spectrophotometrically using the Brucine method (EPA, 1972). Briefly, 10 ml of 10 N sulfuric acid was added to the water sample (10ml) and allowed to cool. 0.5 ml of brucine in sulfanilic acid solution was added to the sample and mixed. The samples were then heated at 100°C in a water bath for 25 minutes. This mixture was then placed in a volumetric flask and diluted upto 25 ml with deionized water. The concentration of nitrates in the sample was then estimated by the absorption of the solution at 410 nm using a spectrophotometer.

For the estimation of phosphates, 25 ml of water sample was added to a volumetric flask, 1 ml of ammonium molybdate was added to it and mixed well. Two drops of stannous chloride was then added to the sample and mixed. The intensity of the blue coloured complex was read spectrophotometrically at 690 nm (Bureau of Indian Standards, 1988).

1.3.6. Total Petroleum Hydrocarbon analysis

Water samples: 2 ml of hexane was added to 20 ml of water and mixed well for 20 min in capped tubes in an orbital shaker. This mixture was allowed to stand for the separation of the two phases. The upper layer containing hexane was collected and dried under a stream of nitrogen gas. The dried sample was then reconstituted with 1.5 ml of HPLC-grade ethanol and 1.5 ml of de-ionized water and stored at 4°C in dim light (Ramachandran *et al.*, 2006). The TPH concentrations were analyzed spectrofluorometrically (Shimadzu RF-5301PC) with excitation at 310 nm and emission at 360 nm. The values were expressed as µg/L.

Sediment samples: Sediment samples were dried in an oven overnight at 70°C. 10 gm of this dried sediment was mixed with tetrohydrofuran, shaken vigorously and placed in an ultrasonicator bath for 1-2 hours. The samples were then centrifuged at 2,000 rpm for 30 minutes and the supernatant was evaporated using a stream of nitrogen gas. The residue was then re-dissolved in 20 ml of hexane and was analyzed spectrofluorometrically with excitation at 310 nm and emission at 30 nm. The values were expressed as µg/g of dry sediment (Telli-Karakoç *et al.*, 2002).

1.3.7. Trace Metal analysis

Water samples: One litre of water was acidified with 1 ml of concentrated HNO_3. One mL of 1% ammonium pyrolidine dithiocarbamate (APDC) and 1 mL of methyl isobutyl ketone

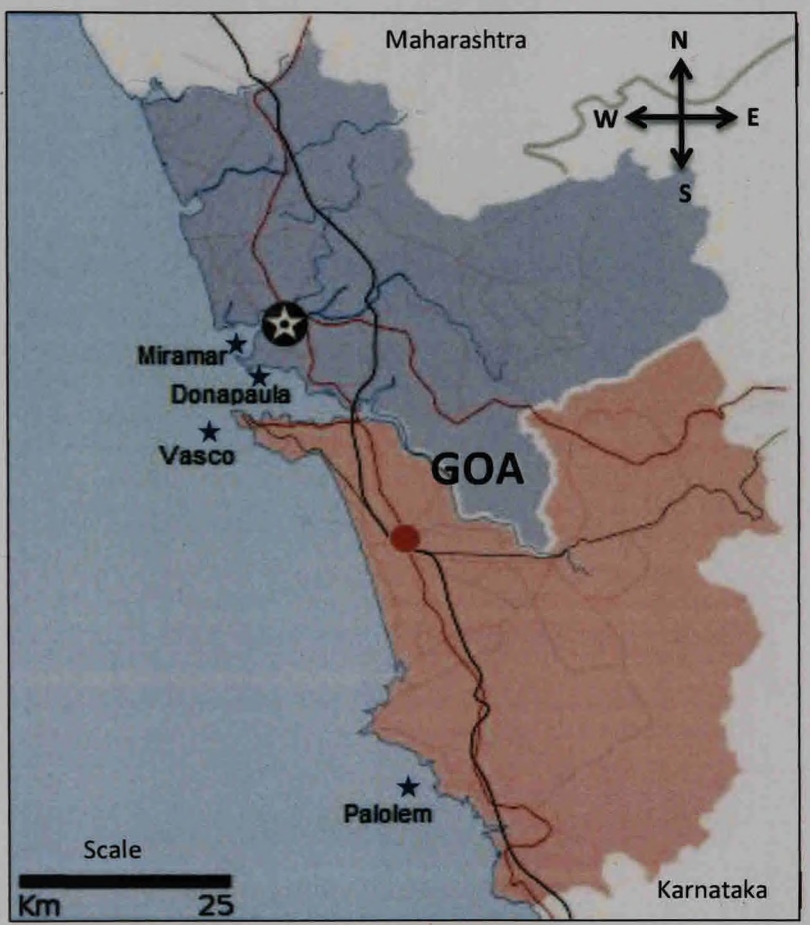
(MIBK) were added to the acidified water in a separating funnel and shaken for 5 minutes. This mixture was allowed to stand for about 15-20 minutes to allow phase separation. The organic layer obtained was transferred to another separating funnel and back-extracted with 20 ml of 4N HNO_3 in another separating funnel. The lower aqueous phase was collected in a centrifuge tube and analyzed for the presence of trace metals such as iron, manganese, copper. cadmium and lead using a flame atomic absorption spectrophotometer (Shimadzu AA-6300) (Satyanarayanan *et al.*, 2007). The concentration of metals was expressed as µg/L of sample.

Sediment samples: 1-2 gm of oven-dried sediment were digested with 10 ml of aqua regia (1:3 concentrated HNO_3 and HCl) and heated on a hot plate at 85°C for 2 hours. The digestate was then diluted to a final volume of 100 ml with distilled water and filtered using a Whatman's filter paper (No. 1) (USEPA, 1996) and analyzed for the presence of trace metals such as iron, manganese, copper, cadmium and lead using a flame atomic absorption spectrophotometer. The concentration of metals was expressed as µg/g of dried sediment.

1.3.8. Statistical analysis

Statistical analysis was performed using IBM© SPSS 23 statistical software package. The data were tested to meet the assumptions of normality using Shapiro-Wilk's test and homogeneity using Levene's test and were subsequently arcsine transformed to improve linearity. These values were then used for various parametric tests. The significance of the data from the polluted sites was evaluated using student's t-test. The results were regarded as statistically significant at $p < 0.001$, 0.01 and 0.05.

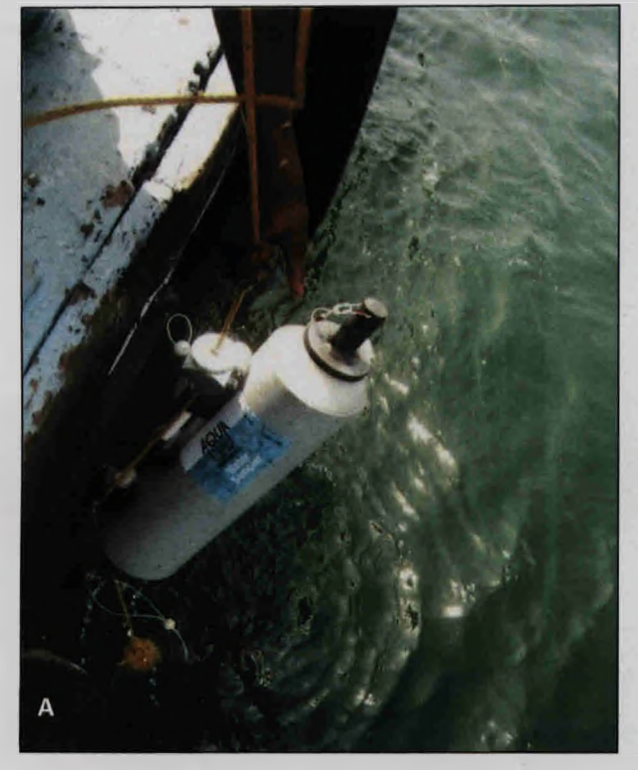
Plate 1.1. Sites selected for the present study along with their coordinates

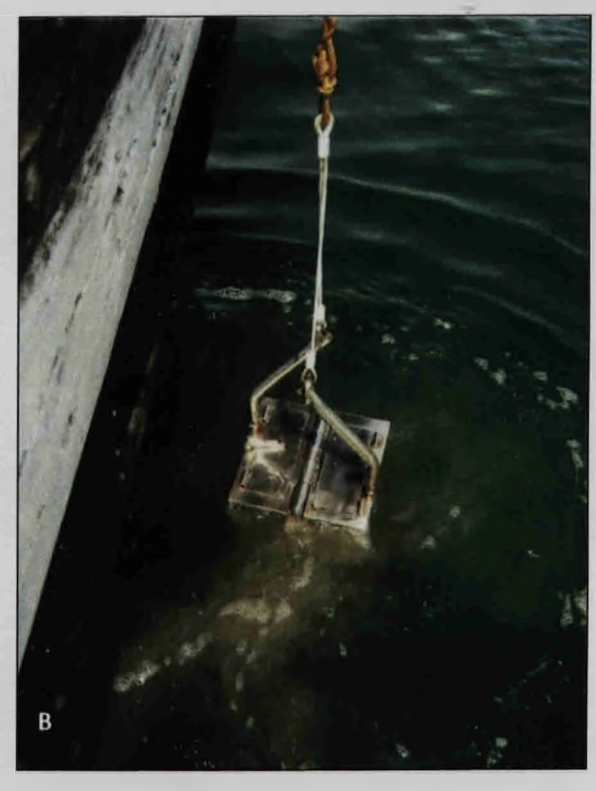


Co-ordinates			
Sr. no.	**Study Site**	**Latitude**	**Longitude**
1	Miramar	15°29'21" N	73°48'18" E
2	Dona Paula	15°46'08" N	73°80'95" E
3	Vasco	15°40'83" N	73°79'16" E
4	Palolem	15°00'17" N	74°02'79" E

Plate 1.2. Trawler operation and fish collection

Plate 1.3. A. Water collection by Niskin sampler and **B**. sediment collection by van Veen grab





1.4. Results

1.4.1. Physico-chemical parameters

The data of the various physico-chemical parameters are represented in figures 1.1 - 1.6. Surface and bottom water temperature ranged between 28 and 30.5°C in the study period extending in all the seasons (fig. 1.1 A and B). Fig. 1.2 A and B depict the pH of the water which ranged between 7 and 8.04 throughout the study period. Salinity was high in both the surface and bottom water of Palolem and decreased during both the monsoon seasons (fig. 1.3 A and B). Dissolved oxygen (DO) level was highest in the waters at Palolem and comparatively low in the waters at Vasco and Dona Paula (fig. 1.4 A and B). The water at Miramar showed intermediate level of DO. Phosphate levels given in figure 1.5.A and B were almost similar at all the sites and did not fluctuate to a large extent with a range between 1.1 to 2.4 µg/L. On the other hand, nitrate levels were found to be high in the surface and bottom waters at Vasco (fig. 1.6 A and B).

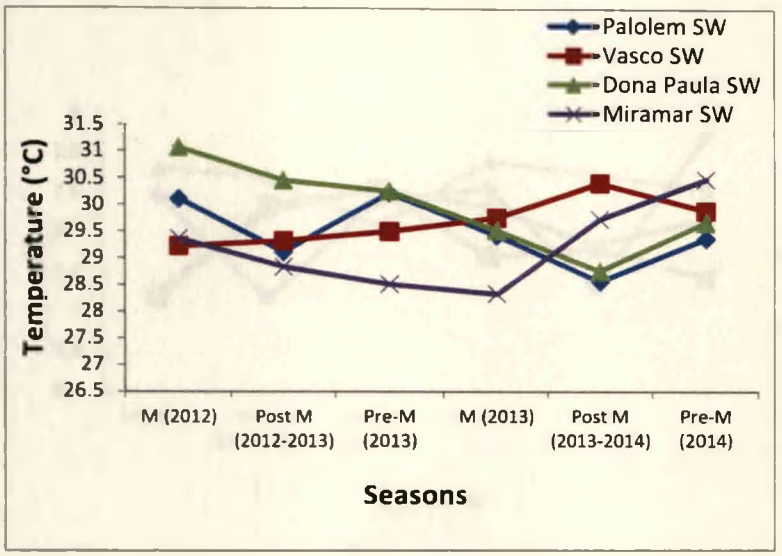
1.4.2. Total petroleum hydrocarbons and trace metals

The concentration of TPH in the surface water, bottom water and sediment from all the sites are represented in fig. 1.7. TPH values were significantly high in both surface and bottom waters of Vasco, Dona Paula and Miramar ($p < 0.001$, $p < 0.01$, $p < 0.05$) in all the seasons compared to that of Palolem. Sediment samples from these sites also contained high concentrations of TPHs compared to that of Palolem. The concentrations of trace metals (Fe, Mn, Cu, Cd and Pb) in the surface and bottom waters as well as the sediment from all the sites are represented in figs. 1.8-1.12. A significant concentration of dissolved Fe was observed in the surface and bottom waters at Vasco and Dona Paula compared to that of Palolem during the monsoon season of 2012 ($p < 0.05$) and was generally non-significant

during the other seasons (fig. 1.8). However, the sediment at Vasco, Dona Paula and Miramar showed significantly high concentrations of Fe in all the seasons ($p < 0.05$). The concentrations of Mn were significant in the water at Vasco during both the monsoon seasons and post-monsoon season of 2013 (fig. 1.9). Mn was also found to be significant in the water at Miramar during the monsoon, post-monsoon and pre-monsoon season during 2012-2014 whereas the concentration of Mn at Dona Paula was found to be non-significant in all the seasons. Cu concentrations (fig. 1.10) were observed to be significant only in the sediment at all the study sites ($p < 0.001$, $p < 0.01$, $p < 0.05$) while its concentrations in water were all non-significant. The surface and bottom water of Vasco and Dona Paula exhibited significantly high concentrations of Cd compared to that of Palolem ($p < 0.05$) whereas that of Miramar was found to be non-significant in all the seasons (fig. 1.11). The concentration of Cd was also found to be elevated in the sediment at Vasco and Dona Paula in all the seasons and even that of Miramar in the post-monsoon of 2012-2013 and the pre-monsoon of 2013-2014. Pb levels were highest in Dona Paula (fig. 1.12). Pb concentrations were found to be consistently high in the sediment of both Vasco and Dona Paula in all the seasons compared to that of Palolem. The sediment at Miramar also exhibited elevated concentrations of Pb in all the seasons except in the monsoon season of 2012. On the other hand, none of the water samples contained significant concentrations of Pb in any season.

Fig. 1.1. Temperature of **(A)** surface water (SW) and **(B)** bottom water (BW) at various study sites in different seasons from June 2012 to May 2014

A



B

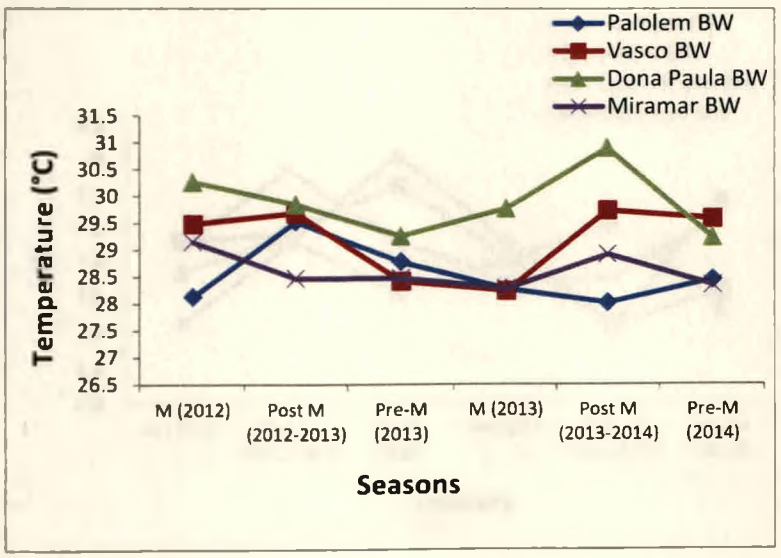
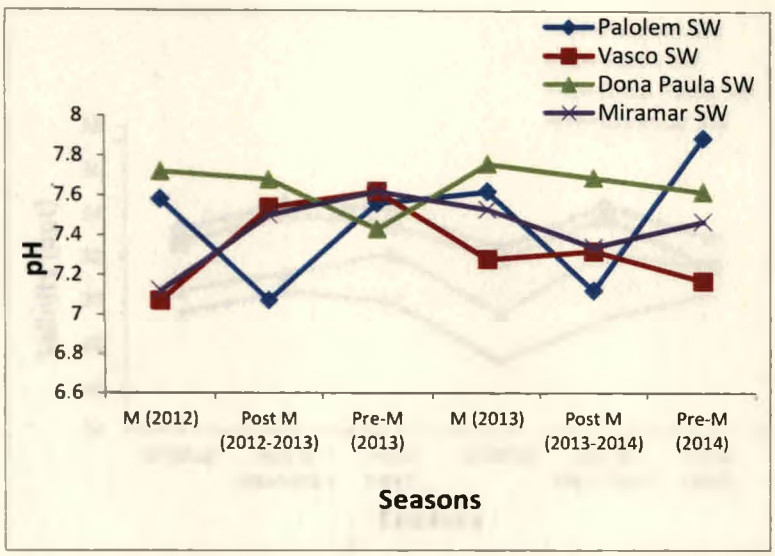


Fig. 1.2. pH of **(A)** surface water (SW) and **(B)** bottom water (BW) at various study sites in different seasons from June 2012 to May 2014

A



B

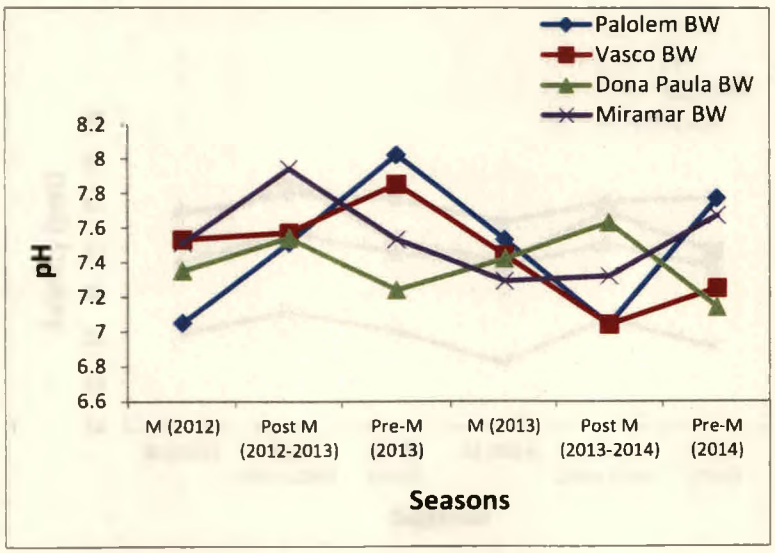
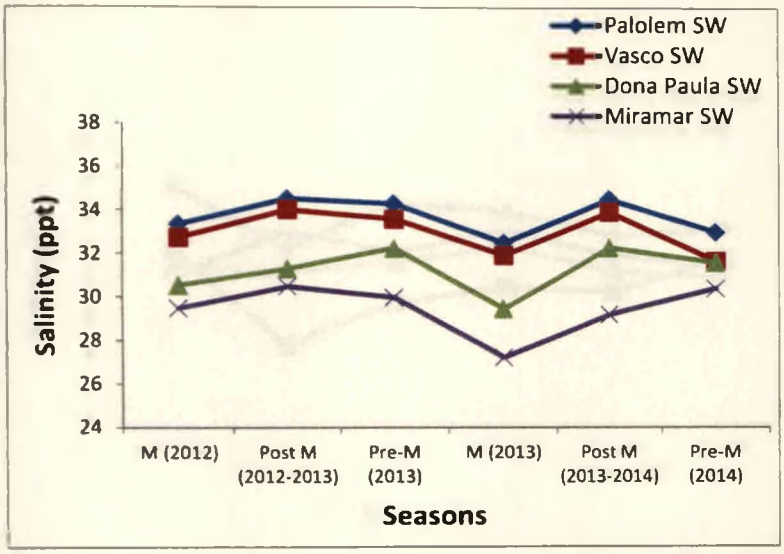


Fig. 1.3. Salinity of **(A)** surface water (SW) and **(B)** bottom water (BW) at various study sites in different seasons from June 2012 to May 2014

A



B

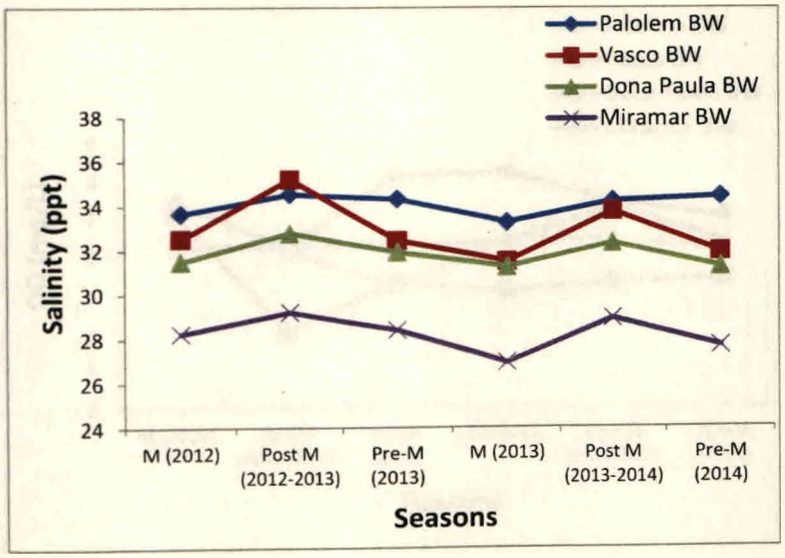
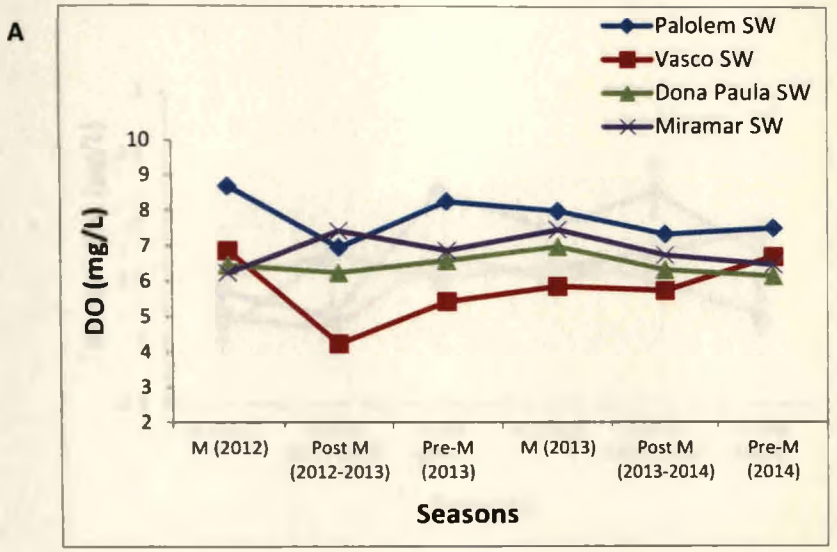


Fig. 1.4. Dissolved oxygen (DO) in **(A)** surface water (SW) and **(B)** bottom water (BW) at various study sites in different seasons from June 2012 to May 2014



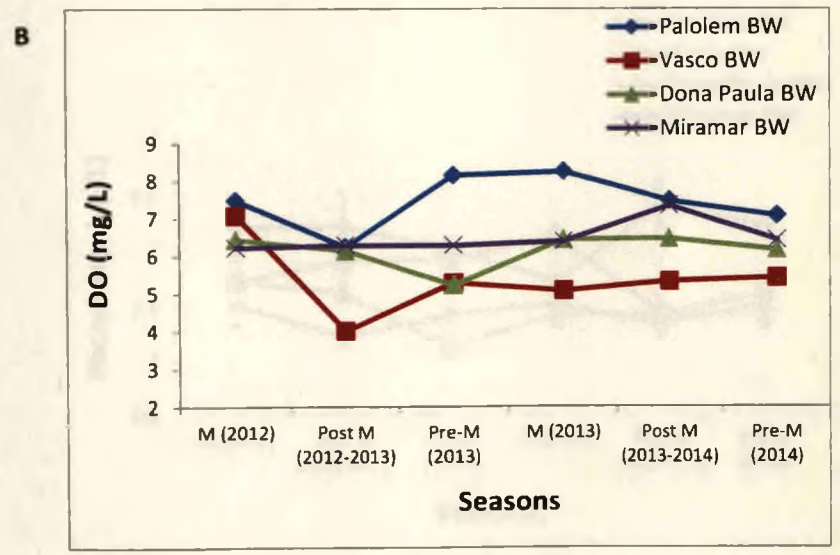
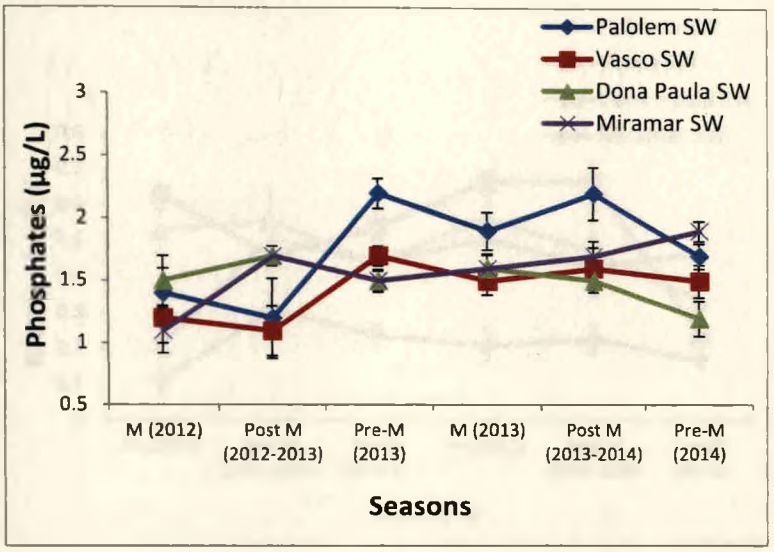


Fig. 1.5. Phosphates in **(A)** surface water (SW) and **(B)** bottom water (BW) at various study sites in different seasons from June 2012 to May 2014

A



B

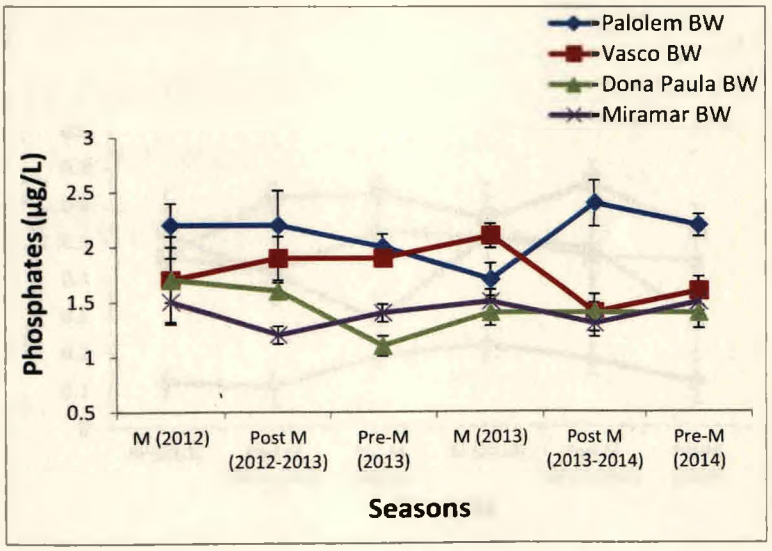


Fig. 1.6. Nitrates in **(A)** surface water (SW) and **(B)** bottom water (BW) at various study sites in different seasons from June 2012 to May 2014

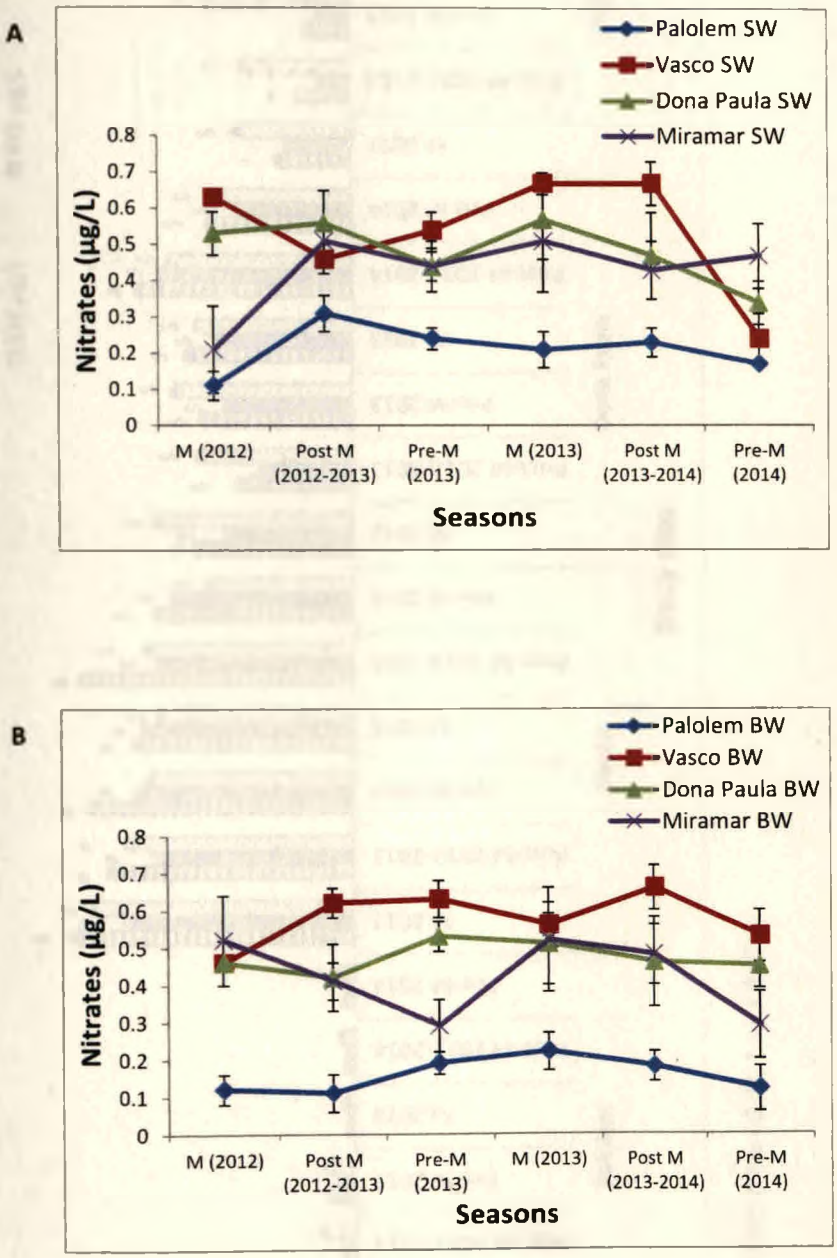
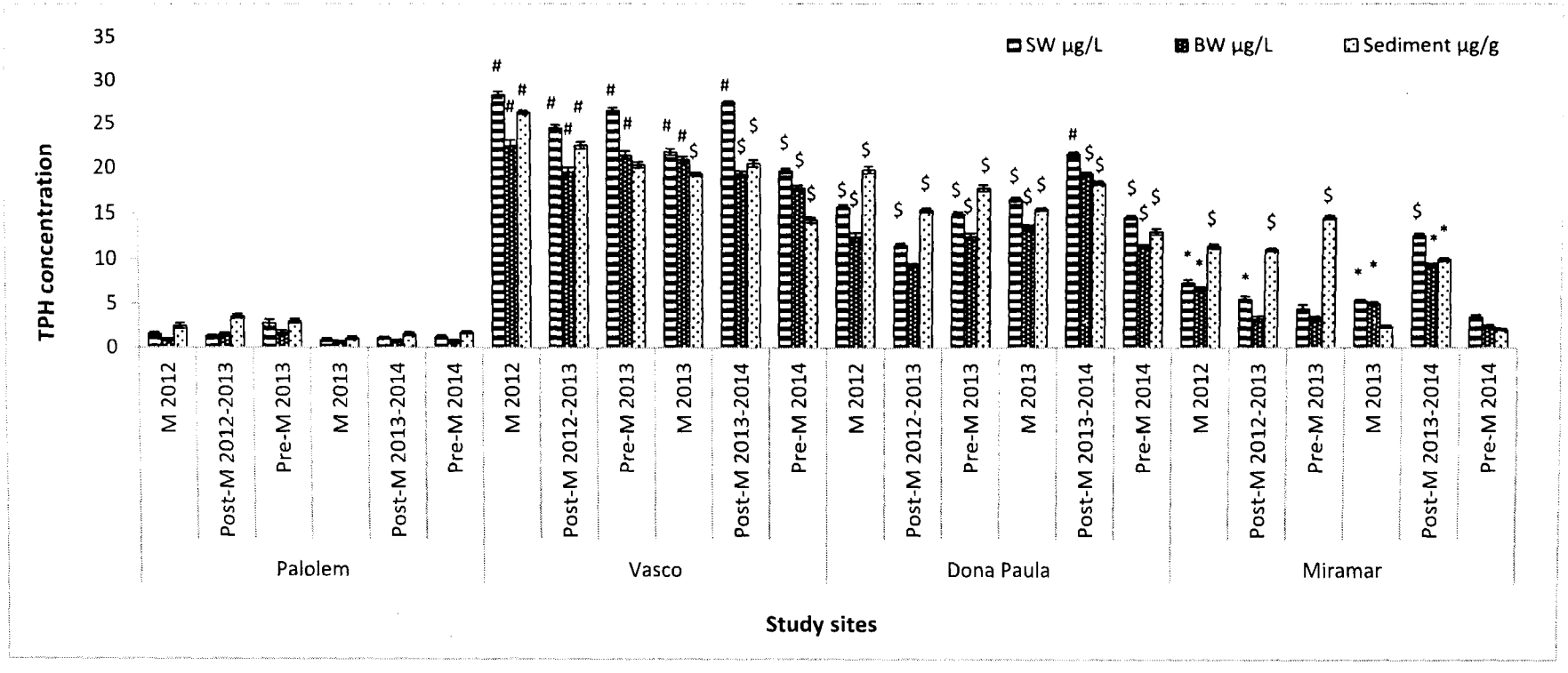
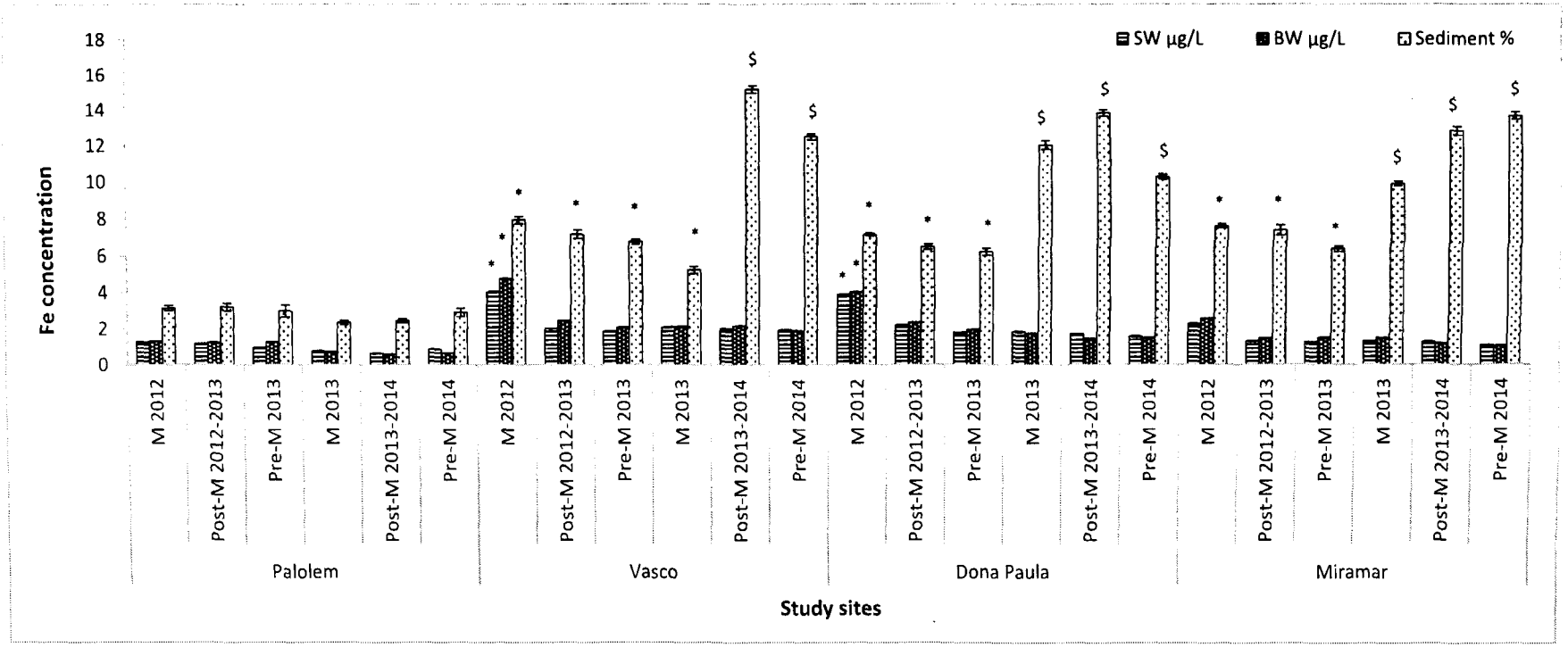


Fig. 1.7. Concentration of TPH in surface water (SW), bottom water (BW) and sediment (Mean ± SD) at various study sites in different seasons from June 2012 to May 2014

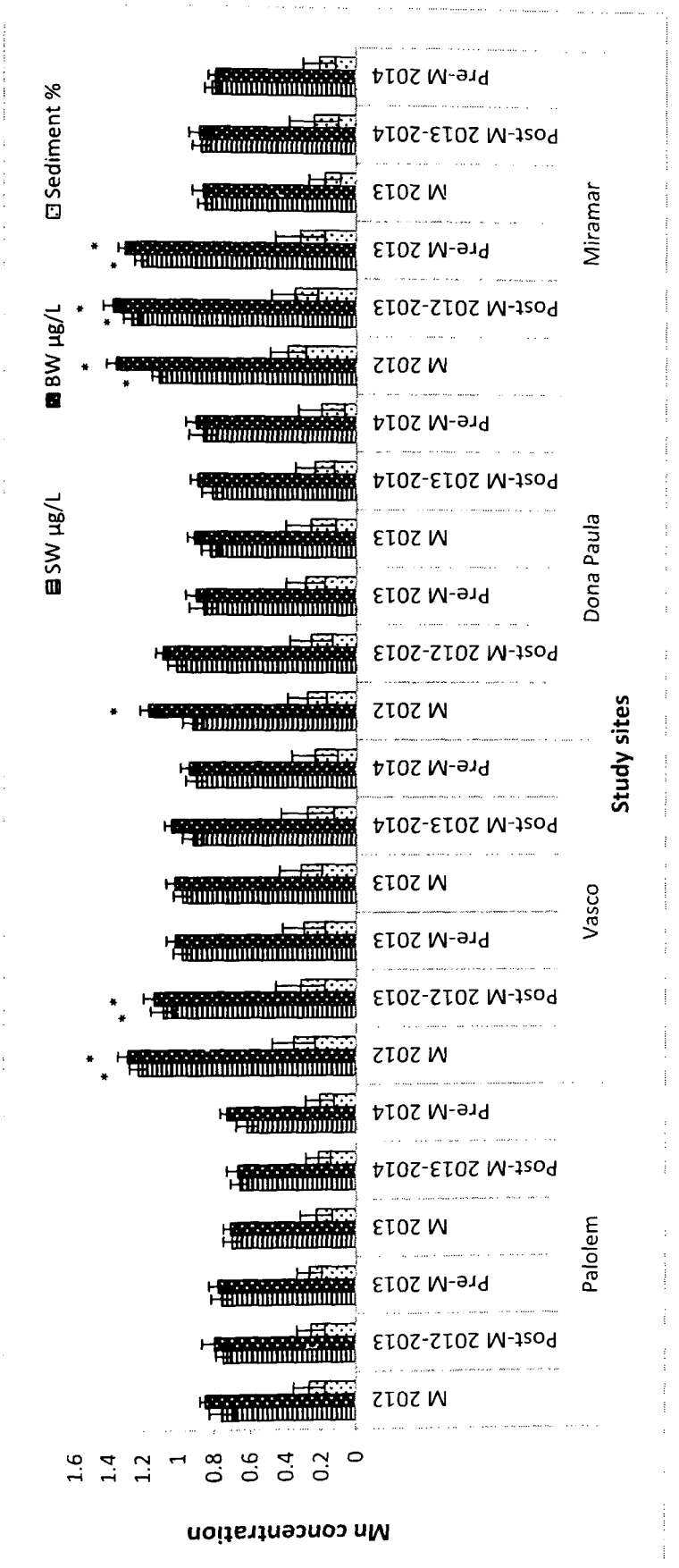


Note: *# = p < 0.001; $ = p < 0.01; * = p < 0.05*

Fig. 1.8. Concentration of Fe in in surface water (SW), bottom water (BW) and sediment (Mean ± SD) at various study sites in different seasons from June 2012 to May 2014



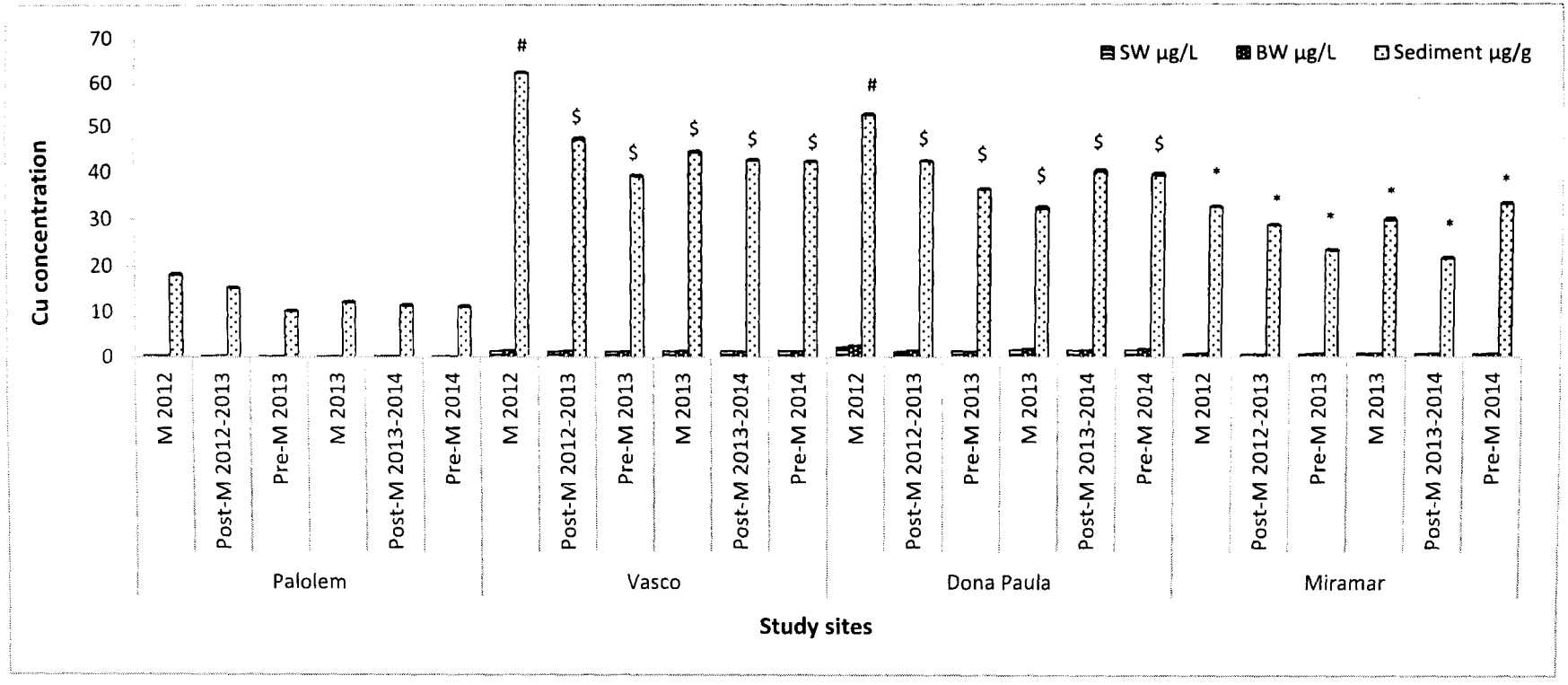
Note: *$ = p < 0.01; * = p < 0.05*



Note: * = $p < 0.05$

Fig. 1.9. *Concentration of Mn in in surface water (SW), bottom water (BW) and sediment (Mean ± SD) at various study sites in different seasons from June 2012 to May 2014*

Fig. 1.10. Concentration of Cu in surface water (SW), bottom water (BW) and sediment (Mean ± SD) at various study sites in different seasons from June 2012 to May 2014



Note: *# = p < 0.001; $ = p < 0.01; * = p < 0.05*

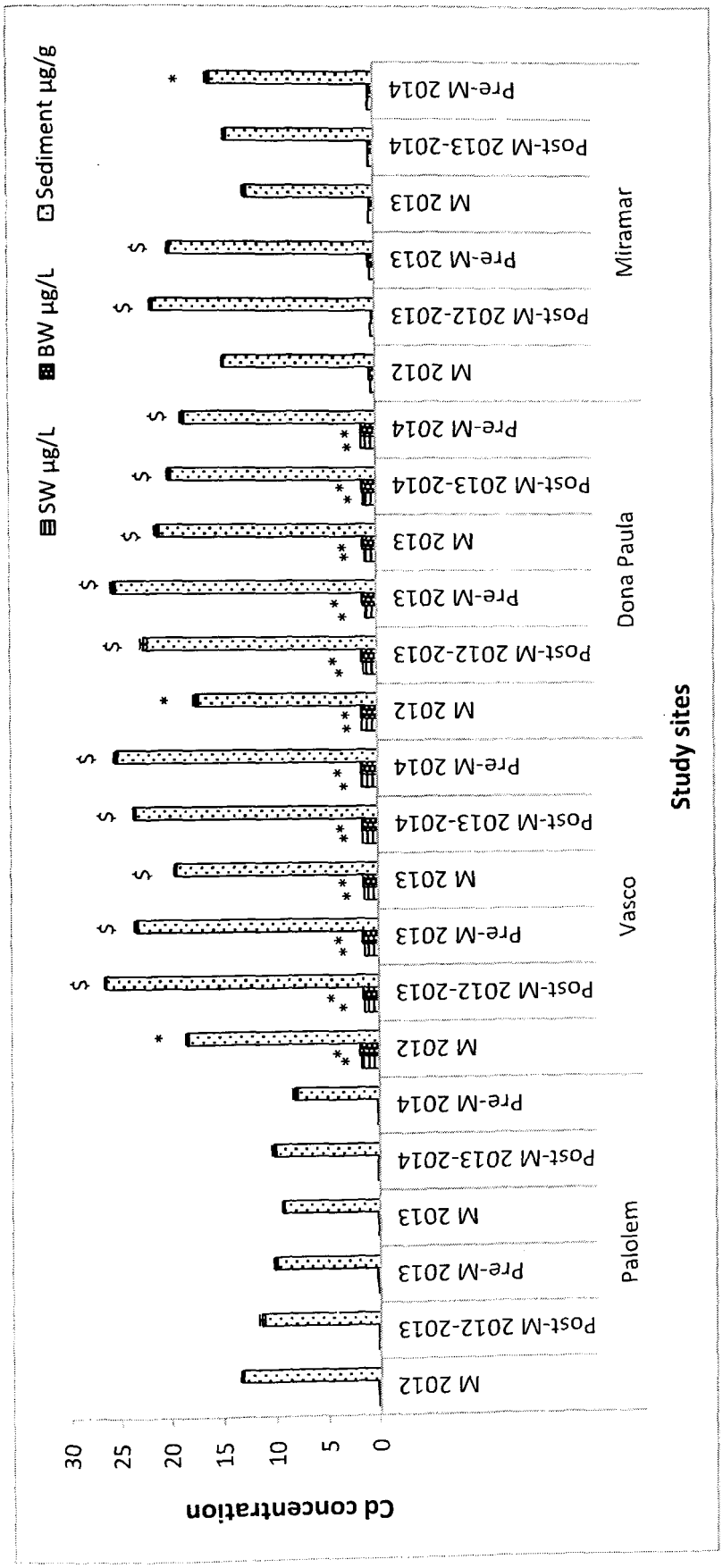
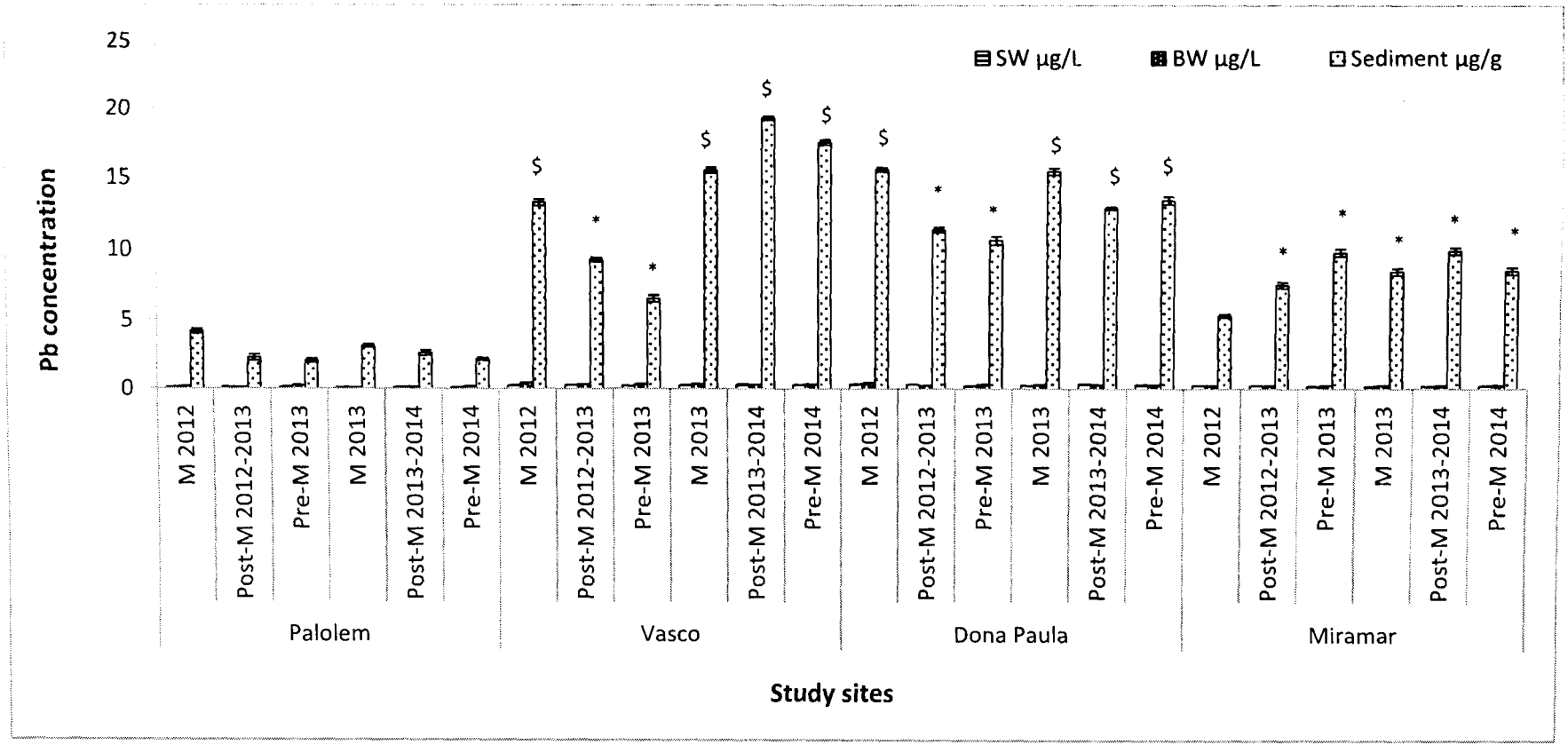


Fig. 1.11. Concentration of Cd in surface water (SW), bottom water (BW) and sediment (Mean ± SD) at various study sites in different seasons from June 2012 to May 2014

Note: $ = $p < 0.01$; * = $p < 0.05$

Fig. 1.12. Concentration of Pb in surface water (SW), bottom water (BW) and sediment (Mean ± SD) at various study sites in different seasons from June 2012 to May 2014



Note: *$ = p < 0.01; * = p < 0.05*

1.5. Discussion

In the present study, the concentrations of both, the heavy metals and the total petroleum hydrocarbons in the water and sediment were found to be significant in Vasco, Dona Paula and Miramar compared to that of Palolem in all the seasons. These pollutants enter the water from diverse sources including mining and associated activities such as ore transportation and loading via the Zuari and Mandovi rivers. The activities from the Mormugao harbor such as ship building and repair may also contribute to the presence of metals and petroleum hydrocarbons at Vasco (Sarkar *et al.*, 2014). The presence of these pollutants is expected to induce a negative impact on the life of flora and fauna of the ecosystems at these sites. Most of the contaminants such as TPHs and heavy metals have a tendency to settle on the surface sediments and thus, the benthic feeders might be affected the most. A wide variety of effects ranging from DNA damage to morphological abnormalities might be implicated as a result of the intake / ingestion of these pollutants by aquatic organisms. Marcon *et al.* (2010) reported that a high content of certain metals in the water and sediment could be one of the factors that might cause genetic damages in fish.

The significant concentrations of TPHs and Fe, Mn, Cu, Cd and Pb in the sediment at Vasco and Dona Paula observed in the present study could lead to DNA damage in aquatic organisms as reported in several studies. Amado *et al.* (2006a) reported significant DNA damage in the Brazilian flounder (*Paralichthys orbignyanus*) from a polluted site in the Patos Lagoon estuary in Southern Brazil compared to an unpolluted reference site, may be because of the contamination with petroleum hydrocarbons and metals such as Cu due to the release of effluents from nearby industries. In another study, Tsangaris *et al.* (2011) also observed DNA damage in the blood and gill cells of the grey mullet (*Mugil cephalus*) from an area

polluted with Cu, Mn, Pb, Cd and PAHs in the Saronikos Gulf in Greece. The presence of heavy metals such as Cu, Cd and Pb primarily due to agricultural activities and discharge of anthropogenic wastes in the water and sediment of the Goksu delta in Turkey were reported to be the cause of DNA damage in catfish (*Clarias gariepinus*), bleak (*Alburnus orontis*) and mullet (*M. cephalus*) (Ergene *et al.*, 2007). These metals were also found to be accumulated in the tissues of these fish. Ahmad *et al.* (2008) and Oliveira *et al.* (2009) observed DNA damage in the form of DNA strand breaks in the gill cells of the European sea bass (*Dicentrarchus labrax*) and the golden grey mullet (*Liza aurata*) captured from Ria de Aveiro which is a polluted coastal lagoon in Portugal. Genotoxicity (nuclear abnormalities) was observed in the peripheral erythrocytes of two fish species (*Limanda limanda* and *Melanogrammus aeglefinus*) from the North Sea and near the coast of Iceland which are highly influenced by ship traffic and oil / gas drilling zones (Baršiene *et al.*, 2013). Genotoxicity was also reported in laboratory hatched Senegalese soles that were placed *in situ* on sediment contaminated with PAHs and heavy metals in the Sado estuary in Portugal (Costa *et al.*, 2011). In another study from Brazil, Katsumiti *et al.* (2009) reported significant DNA damage in the native species of catfish (*Cathrops spixii*) in a bay south of Brazil after an accidental explosion of an oil tanker which released large amounts of bunker oil.

Besides in fishes, a number of effects of these pollutants have also been reported in bivalves. Pisanelli *et al.* (2009) reported seasonal variability of DNA damage in the hemocytes of *Mytilus galloprovincialis* which was found to be correlated with the seasonal fluctuations of trace metals in the Adriatic Sea. In a study conducted by Magni et al. (2006), mussels (*Mytilus galloprovincialis*) were from a commercial port in Italy and were reported to have significant DNA damage in them compared to the bivalves collected from a reference site.

This DNA damage was found to be attributed to the elevated concentrations of heavy metals such as Pb and Cd in the sediment at the port compared to the reference site. The comet assay was also used to detect DNA damage in the gill cells and hemocytes of *Mytilus edulis* sampled from coastal waters which are suspected to be polluted with metals and petroleum hydrocarbons from the Køge Bay in Denmark (Rank *et al.*, 2005). This DNA damage was further found to be correlated with the concentrations of heavy metals such as Ni, Cd, Cr and Hg in the sediment from the polluted sites. In another study, Manila clams (*Tapes semidecussatus*) were chronically exposed to sediments contaminated with PAHs and metals such as Cu, Pb, Cd and Zn and were found to exhibit significant DNA damage compared to clams that were exposed to sediment from a pristine location (Coughlan *et al.*, 2002). DNA damage was also reported in zebra mussels (*Dreissena polymorpha*) sampled along a contaminant gradient in the St. Lawrence River in Canada (de Lafontaine *et al.*, 2000). The river received considerable pollutants such as effluents from paper mills and metals, PAHs and PCBs from ship related activities which could have induced genotoxicity in these zebra mussels. Sediments collected from a polluted area in the Seine estuary, France were organically extracted and the toxicity of these extracts and were exposed to the embryos of *Crassostrea gigas*. The extracts which were found to contain high levels of PAHs and PCBs were able to induce significant abnormalities in these embryos than the extracts from a pristine location (Cachot *et al.*, 2006). Fu *et al.* (2013) observed an increase in the expression of genes related to DNA damage responses in fish collected from the Yangtze River in China which was attributed to the high concentrations of heavy metals in the water and sediment.

1.6. Conclusion

Petroleum hydrocarbons and heavy metals such as Fe, Mn, Cu, Cd and Pb are present at significant concentrations in the water and sediment at Vasco, Dona Paula and Miramar compared to that of Palolem in all the seasons. The presence of these pollutants could pose a threat to the finfish and shellfish inhabiting these waters as reported by numerous researchers earlier. Further analyses of the water column and sediment could supplement our findings and provide a broader perspective of the overall "health" of the aquatic ecosystems at these sites.

Chapter 2:

Mutagenic pollutants and genetic damage in the fauna of the Goan coast

2.1. Introduction

The rivers Mandovi and Zuari are the most important navigable rivers of Goa. They originate in the Western Ghats and flow through the state of Goa to join the Arabian Sea. These rivers inevitably receive and carry diverse kinds of contaminants from upstream mining sites, and deposit them in the coastal waters of Goa. Besides this input, the coastal water also receives large quantities of waste water in the form of sewage from various sources (i.e. industrial, agricultural, domestic, etc.) which induce considerable marine pollution. A majority of the pollutants are reported to be toxic to the marine organisms and other associated organisms including fish and shellfish, which form a major group of the economically important seafood of Goa.

Sea food is a major route of exposure of human populations to toxic chemicals. Finfish and shellfish have been recognized as major mediators for contaminant transfer to humans and a cause of innumerable sufferings in man (Al-Sabti and Metcalfe, 1995). Genotoxic / mutagenic compounds, including carcinogens, can manifest as components of complex mixtures in the environment that can have adverse health effects on indigenous biota as well as humans (Dearfield *et al.*, 2002).

Genotoxic effects in fishes may induce deleterious cellular changes leading to various diseases including cancer, reduced reproductive competence, teratogenicity and even may be fatal, resulting in low fish catch. Further this will also affect the normal food chain and result in a deleterious shift in the biotic community of this coastal ecosystem. These will result in a significant reduction in the quality / palatability of the sea food. The uptake of xenobiotic contaminants, such as PCBs and PAHs, is highly detrimental to the metabolic activity as well

as the biological integrity of marine organisms. Many of these pollutants are chemical carcinogens and / or mutagens with the capacity to induce various types of DNA damage.

Biomonitors / bioindicators are organisms which, by their presence or absence indicate the presence of a particular critical factor such as environmental stressors (Martin and Coughtry, 1982). All organisms can be considered as bioindicators depending upon their sensitivity to this critical factor within a particular location (Phillips and Rainbow 1994). Although mostly the genotoxic effects induced by exposure to these stressors may be compensated by DNA repair mechanisms as a natural phenomenon, signs of toxicity will most likely be evident at the upper limits of tolerance. Bioindicators are key for assessing the level of environmental pollution and are therefore employed at sites of anthropogenic stress (Phillips and Rainbow 1994).

2.1.1. Fishes as bioindicators

Fishes play a very important role as consumers in an aquatic ecosystem. Further, they exhibit the intake and accumulation of many of the pollutants of the aquatic environment and thereby contribute to their bioaccumulation / biomagnification through the food chain. In aquatic ecosystems, pollutants may accumulate in sediment as well as the organisms at different trophic levels in this food chain, including the benthic and pelagic animals. Fishes are the most predominant pelagic animals in an aquatic environment representing the primary / secondary / tertiary consumers, whereas, bivalves are mostly sedentary in nature and are filter feeders. Fishes being at the higher tropic level of aquatic food chain can readily accumulate a variety of contaminants by ingestion of smaller species, as well as, through water intake. They also respond to mutagens at fairly low concentrations and are more sensitive to the induction of genetic damage. Man, being at the apex of this food chain

consumes varieties of fishes and bivalves as sea food and becomes the final recipient of these pollutants. Fishes are therefore the most popular animals used as bioinidicators of genotoxic agents in the aquatic environment as they are economically important food sources, higher order predators, form a major part of aquatic communities and are sensitive to the effects of pollution and capable of concentrating xenobiotics (Kligerman, 1982). They can therefore act as "sentinel" organisms for indicating the potential for exposure of human populations to genotoxic chemicals in drinking water.

2.1.2. Bivalves as bioindicators

Bivalves are widely used as bioinidicators for coastal pollution monitoring programs (Krishnakumar *et al.*, 2006). Many field studies have shown that some of these biomarkers of environmental contaminants in mussels can be used to discriminate between polluted and unpolluted sites (Krishnakumar *et al.*, 1994; 1995). The use of molluscs, particularly bivalves as sentinels is a popular approach for detecting various kinds of contamination in the environment (Zuykov *et al.*, 2013). Bivalves are widely used in biomonitoring programs due to their association with the estuarine sediment, filter-feeding habit and ability to bioaccumulate various contaminants. A number of bivalve species such as *Ruditapes phillipinarium* and *Mytilus galloprovincialis* which are routinely used for these programs are usually natural populations or cultured and transplanted at areas suspected of pollution (Bolognesi *et al.*, 2004; Vlahogianni *et al.*, 2007; Moschino *et al.*, 2012; Ji *et al.*, 2015).

2.1.3. Accumulation of metals and petroleum hydrocarbons

Fishes and bivalves are known to accumulate pollutants such as heavy metals and petroleum hydrocarbons in their tissues to concentrations several times higher than that of the ambient water (National Research Council, 1991; Schwacke *et al.*, 2002). In non-polluted

circumstances, concentrations of these chemicals in the tissues of aquatic organisms are often in equilibrium with the natural concentrations in the water. Thus, the tissues of aquatic organisms contain natural background concentrations of many naturally-occurring chemicals such as most metals and aromatic hydrocarbons. These background body concentrations of chemicals are probably not toxic to the aquatic organisms. However, increased inputs to the environment of some of these chemicals from anthropogenic activities can result in increase of their concentration in the water and also enhanced bioaccumulation in the tissues of organisms, possibly to concentrations that are toxic to the organisms themselves or their consumers, including man. The estuarine and coastal waters of Goa receive pollutants at much higher than normal background levels from sources such as mining sites, industries, ships and harbor related activities. The fauna inhabiting these water bodies inevitably take up these pollutants from the water and are often unable to efficiently excrete them out of their bodies leading to the bioaccumulation of these chemicals in their tissues.

2.1.4. Genotoxicity tests

Micronucleus test

Micronuclei (MNi) result either from acentric chromosomal fragments or whole chromosomes lagging behind during metaphase/anaphase transition induced by clastogens or by spindle dysfunctions, respectively and are not integrated into the daughter nuclei. These MNi harbouring chromosomal fragments result from direct DNA breakage, replication on a damaged DNA template and inhibition of DNA synthesis. MNi consisting of whole chromosomes are primarily formed from the failure of the mitotic spindle polymerization, kinetochore or by damage to chromosomal structures, alterations in cellular physiology and mechanical disruption (fig. 2.1.). Thus, an increase in the frequency of micronucleated cells

is a biomarker of genotoxic effects that can reflect exposure to agents with clastogenic or aneugenic (aneuploidogenic) modes of action.

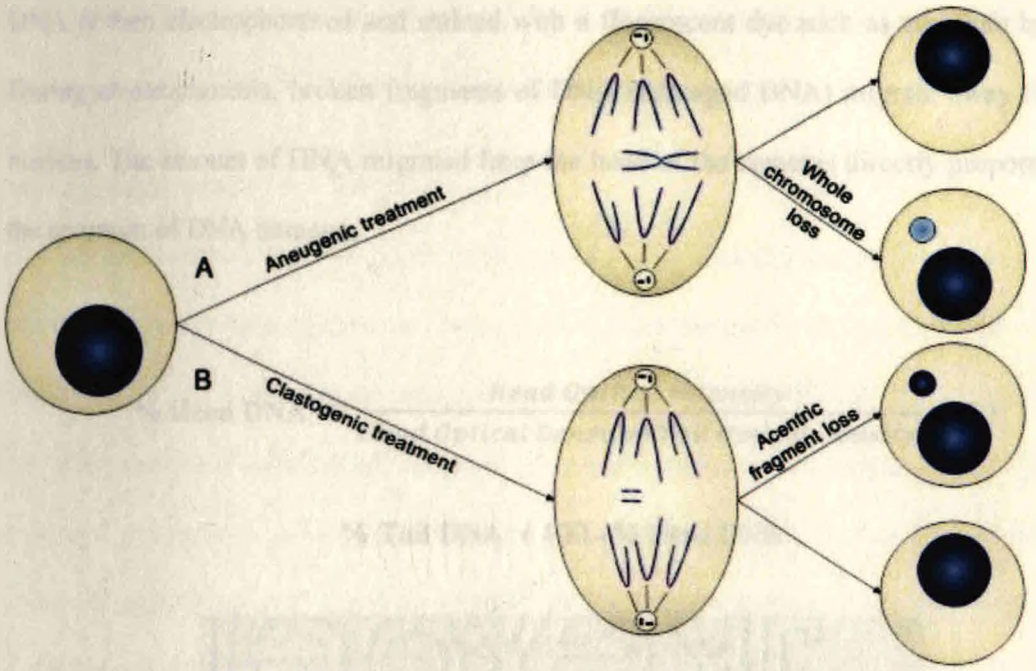


Fig. 2.1. Mechanism of formation of micronuclei.

Source: *Terradas et al. (2010). Genetic activities in micronuclei: Is the DNA entrapped in micronuclei lost for the cell? Mutation Research/Reviews in Mutation Research. 705: 60-67*

Alkaline Single-cell gel electrophoresis (Comet assay)

The alkaline single cell gel electrophoresis or comet assay is a simple, rapid, visual and sensitive technique for analyzing and quantifying DNA damage (induced by genotoxic agents even at low concentrations) in any eukaryotic cell, reflected as single strand breaks under alkaline conditions. A damaged cell in the comet assay has the appearance of a "comet" with a fluorescent intensity that is directly proportional to the number of DNA strand breaks present. DNA that is undamaged will appear as intact comet heads without tails after electrophoresis. In the comet assay, the cells are embedded in a thin layer of agarose gel

on a microscope glass slide. The cells are then subject to lysis to remove all cellular proteins and the DNA is allowed to unwind under alkaline conditions. Following unwinding, the DNA is then electrophoresed and stained with a fluorescent dye such as ethidium bromide. During electrophoresis, broken fragments of DNA (damaged DNA) migrate away from the nucleus. The amount of DNA migrated from the head of the comet is directly proportional to the quantum of DNA damage.

$$\textbf{\% Head DNA} = \frac{Head\ Optical\ Intensity}{Head\ Optical\ Density + Tail\ Optical\ Density} \times 100$$

$$\textbf{\% Tail DNA} = 100 - \%\ \text{Head DNA}$$

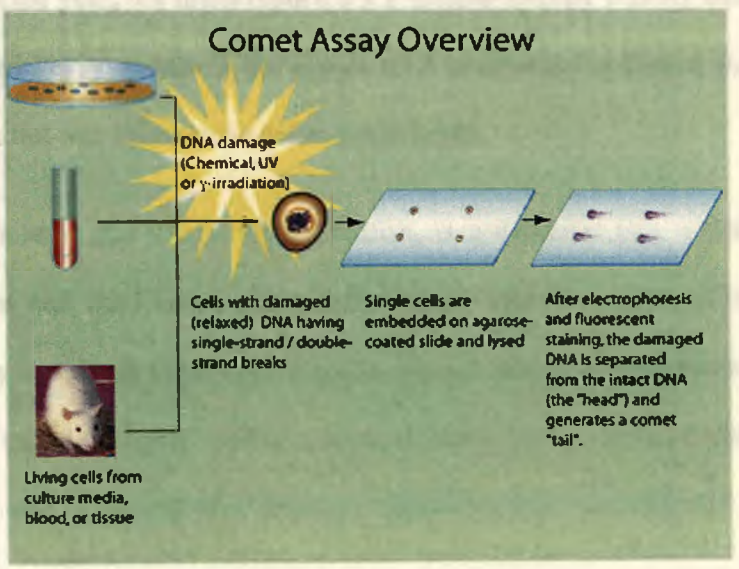


Fig. 2.2. Overview of the comet assay.

Source: *https://www.sigmaaldrich.com/life-science/cell-biology/cancer-research/learning-center/cancer-research-protocols/comet-assay.html*

Both, the MN test and comet assay are routinely used as biomarkers for monitoring aquatic pollution by genotoxic contaminants and can be combined with other physiological and biochemical biomarkers to fully assess the pollution status of various water bodies (Bolognesi and Cirillo, 2014; Praveen Kumar *et al.*, 2015).

2.1.5. Animals selected for the present study

Johnius dussumieri (Cuvier) or Sin croaker (due to the croaking sound it makes when the abdominal muscles beat against the swim bladder) is a Sciaenid fish that is found in Indian coastal waters and often enters estuaries (Talwar and Jhingran, 1991). This species along with other species of croakers are collectively called "*dodyaro*" in Konkani and are used in a variety of dishes in coastal cuisine (Plate 2.1 A). Although *J. dussumieri* is consumed by Goans throughout the year, not many reports are available on their toxicity studies. Singbal *et al.* (1982) reported heavy metals in the tissues of *J. dussumieri* collected from the Aguada Bay in Goa, India but was found to be below toxic limits.

Arius arius (Hamilton), the Threadfin sea catfish, is a demersal fish which is commonly found in estuaries and tidal rivers along both the east and west coast of India (Jayaram, 1984). It is easily caught in trawl nets, bagnets, dipnets and even with hook and line. This catfish is locally called "sangott" and is consumed either fried or in Goan fish curries (Plate 2.1 B).

Meretrix casta (Chemnitz), which is commonly called the backwater or estuarine clam is usually found in the backwaters or estuaries of both the east and west coast of India (Seshappa, 1971). It is a commercially important bivalve species that is consumed as a local delicacy in many parts along the coast of Goa and is available throughout the year (Plate 2.2

A). A few toxicity studies are available on *M. casta* despite its consumption by a majority of the coastal states of India. Sophia and Balasubramanian (1992) observed changes in the condition indices of *M. casta* exposed to water soluble fractions of diesel, engine oil and Kuwait crude oil for a period of 30 days. *M. casta* exposed to various doses of γ-radiation exhibited single-strand breaks in DNA of their hemocytes as measured by the single cell gel electrophoresis (comet) assay (Praveen Kumar *et al.*, 2014).

Meretrix ovum (Hanley) also known as the estuarine backwater clam occurs in abundance in the backwaters of the west coast of India and is also consumed by the local people of Goa (Nayar and Mahadevan, 1974) (Plate 2.2 B). As with *M. casta*, very few studies have been undertaken on this clam. Revankar and Shyama (2009) reported the genotoxic effects of various doses of an organophosphate pesticide, monocrotophos on *M. ovum* using the micronucleus test.

2.2. Review of Literature

A number of fish species have been used as potential bioindicators of pollution in their respective aquatic habitats. Amado *et al.* (2006b) used the croaker, *Micropogonias furnieri* as a bioindicator species for analyzing the pollution in an estuary in Brazil. They observed significant DNA damage in these fish using the micronucleus test and comet assay which was also found to be dependent on seasonal variation in the estuary. The micronucleus test has also been used effectively to monitor the genotoxic potential of pollutants such as Cu, Cd, Ni and Pb in three species of fish (*Clarius gariepinus*, *Alburnus orontis* and *Mugil cephalus*) from the Goksu delta in Turkey (Ergene *et al.*, 2007). A number of studies are also available on the genotoxicity in fish from the North Atlantic Ocean and Baltic Sea in Europe. Baršiene *et al.* (2004) reported significant DNA damage as % MN in the peripheral blood of

flounder (*Platychthys flesus*) from an area of the Baltic Sea which received contaminants from municipal sewage discharges and an oil refinery plant. This DNA damage was also found to be correlated with PAH metabolites in the liver of the fish indicating the genotoxic potential of PAHs. Significant MN was also found to be induced in the erythrocytes of flounder (*P. flesus*), dab (*Limanda limanda*) and cod (*Gadus morhua*) which are native fish species from the Baltic sea and North sea (Rybakovas *et al.*, 2009). This induction of DNA damage was found to be attributed to the presence of PAHs, heavy metals and other pollutants such as polychlorinated biphenyls (PCBs). Polycyclic aromatic hydrocarbons and heavy metals were found to be bioaccumulated in the tissues of the European eel (*Anguilla anguilla*) collected from a coastal region in Western Europe (Oliveira-Ribeiro *et al.*, 2005). This accumulation led to the formation of lesions and tumours particularly in the spleen and the liver which could have resulted as a consequence of DNA damage. Dévier *et al.* (2013) reported DNA damage as DNA strand breaks in the blood cells of dab (*L. limanda*) from the Seine estuary in France. They further reported a significant correlation between this DNA damage and PAH concentration from the biliary metabolites of the fish. DNA damage was also found to be correlated with the concentrations of heavy metals such as Fe and Cu in the livers of grey mullets from a polluted bay in Egypt (Aboul-Ela *et al.*, 2011). Baršiene *et al.* (2015) reported DNA damage in the form of micronuclei in the peripheral blood of three fish (flounder *Platichthys flesus*, herring *Clupea harengus*, and eelpout *Zoarces viviparous*) collected from an area polluted with PAHs and heavy metals in the Baltic Sea. DNA damage was also reported in two fish species (*Mugil cephalus* and *Dicentrarchus labrax*) collected from the Bizerte Lagoon from the Mediterranean Sea (Ameur *et al.*, 2015). This lagoon is reportedly polluted by industrial wastes, and chemical pesticides and fertilizers through

runoffs and is the considered to be the reason for the DNA damage in these fish. Recently, Gusso-Choueri *et al.* (2016) also reported significant genotoxicity in the form of % tail DNA and % MN in the peripheral blood of a marine catfish (*Cathorops spixii*) collected from a mining-affected area in Brazil. They also reported that this genotoxicity was associated with heavy metals and PAH metabolites in the livers of the fish.

The significance of using clams as sentinel species has been demonstrated by Fernández-Tajes *et al.* (2011) wherein they used three species of bivalves (*Venerupis pullastra*, *Cerastoderma edule* and *Mytilus galloprovincialis*) for the biomonitoring of an estuary in the North-West of Spain. DNA damage using the comet assay was found to be correlated with the pollution loads and they further reported that clams and cockles were more sensitive to the effects of pollution. DNA damage was also reported as % MN and DNA adducts in the gill cells of *Mytilus galloprovincialis* collected from a polluted location in the Venice Lagoon, Italy and were found to be correlated with tissue concentrations of metals such as As, Cd, Ni and Pb as well as polycyclic aromatic hydrocarbons (Venier and Zampieron, 2005). Dallas *et al.* (2013) observed genotoxic and cytotoxic effects in two species of bivalves (*Cerastoderma edule* and *Mytilus edulis*) sampled from polluted areas along Tamar estuary in England. Further, a positive correlation was also found between the metal concentration in the soft tissues of the bivalves and that of the sediment. In another study, Jebali *et al.* (2007) reported an increased DNA damage in the clam, *Ruditapes decussatus* collected from polluted sites along the coast of Tunisia. DNA damage was also found to be significantly high in the mussel, *Unio pictorium* which were transplanted in a metal polluted river in Italy (Guidi *et al.*, 2010). Bellas *et al.* (2007) also deployed caged mussels (*Mytilus edulis*) for 30 days at an estuary in Sweden during dredging operations

which resulted in petroleum contamination. They observed a correlation between tissue concentrations of PAHs, metals and DNA damage as % tail DNA indicating the genotoxic potential of these bioaccumulated pollutants. Tissue concentrations of Cu and Zn were found to be significantly high in the soft tissues of oysters (*Crassostrea angulata*) and mussels (*Mytilus galloprovincialis*) sampled from the South Atlantic ocean (Funes *et al.*, 2005). It was also observed that DNA damage occurring as DNA adducts in the tissues of these bivalves could have arisen as a consequence of accumulation of these metals. A multibiomarker approach using genotoxicity assays along with physiological and biochemical assays could be used to potentially assess the quality of water and sediment at polluted sites (Bolognesi and Cirillo, 2014). Gutiérrez *et al.* (2018) observed DNA damage as % tail DNA and % MN in the hemocytes of the brown mussels (*Perna perna*) collected from a coastal zone influenced by an offshore oil platform in southeastern Brazil. Further, the DNA damage was found to be significant in the mussels collected from this site compared to an unpolluted reference site. DNA damage in the form of % tail DNA was also reported in the mussel *Mytilus galloprovincialis* collected from polluted bays in the Aegean Sea (Kacar *et al.*, 2016). Further analyses of the hemolymph and gills revealed the presence of PAHs and heavy metals which correlated with the DNA damage observed in the mussels. In another similar study, mussels (*P. virdis*) sampled from the Ennore Estuary in Chennai, India were observed to exhibit significant DNA damage (% tail DNA) in the gills and hepatopancreas (Vasanthi *et al.*, 2017). Additionally, this damage was found to be positively correlated with the concentration of heavy metals in the soft tissues of the mussels.

A study by Sarkar *et al.* (2014) on the marine gastropod *Morula granulata* at different locations along the Goan coast revealed that the highest DNA integrity was observed at

Arambol (North Goa), identified as the reference site, whereas the lowest DNA integrity was observed at Hollant (South Goa) which is situated between the two most contaminated sites at Bogmalo and Velsao. Similarly the low integrities of DNA in *Cronia contracta* at Vasco, Dona Paula and Velsao sites can be attributed to exposure to genotoxic contaminants especially polycyclic hydrocarbons (PAHs) and toxic heavy metals (Pb, Cd, Cu, Fe and Mn) prevalent in the marine environment as evident by their accumulation in the tissues of the marine snails inhabiting different sites along the Goa coast (Sarkar *et al.*, 2008). The study of DNA integrity in marine snails (*Planaxis sulcatus*) of the Arabian Sea along the Goan coast provides an excellent tool for evaluation of the genotoxic effects of pollution in coastal regions (Sarkar *et al.*, 2006).

2.3. Materials and Methods

2.3.1. Study sites

Vasco, Miramar and Dona Paula were selected as the probable polluted sites and Palolem was selected as the unpolluted (reference) site (Fig. 1) as per the details stated in chapter 1.

2.3.2. Sampling seasons

Sampling was carried out seasonally for a duration of two years as mentioned in chapter 1 (see 1.3.1).

2.3.3. Sample collection

Fish collection: *Arius arius* (Hamilton 1822) (Thread-fin sea catfish) and *Johnius dussumieri* (Cuvier 1830) (Sin croaker) were selected for the present study due to various reasons: (i) their demersal habitat (ii) availability in Goan waters throughout the year and (iii) their consumption by a majority of people living along the coast. Fishes were collected with the

help of a trawl net (mesh size 40 mm), which was mechanically operated by a trawler at the sites as described for water and sediment collection. Blood was immediately drawn from the caudal vein, smeared on clean glass slides and stored in slide boxes. Excess blood was stored in microfuge tubes containing phosphate buffered saline (pH 7.4) at 4°C. The fishes were then stored in clean labelled polythene bags and transported in ice bags to the laboratory for further analyses of the metal and total petroleum hydrocarbon content in their tissues.

Bivalve collection: *Meretrix casta* (Chemnitz 1782) and *Meretrix ovum* (Hornell 1917) (Estuarine backwater clams) were selected for the present study because they are consumed by a majority of the coastal population and are also available in the Goan estuaries throughout the year. The bivalves (both sexes) were collected from the intertidal zone with the help of skilled local fishermen from Palolem and from an estuarine location in Vasco. They were stored in a bucket with water from the study site and transported alive to the laboratory.

2.3.4. Quality assurance and quality control

The appropriate quality assurance methods of sample preparation, handling and preservation were carried out in accordance with US EPA procedures. Bombay High crude oil, a certified reference material was used for TPH estimation and was also used to prepare the calibration curve. "TraceCERT certified reference metals AAS (Sigma)" were used for trace metal estimation.

2.3.5. Micronucleus Test

The micronucleus test was carried out as detailed by Baršiene *et al.* (2004; 2006a). Blood was collected from the caudal vein of fish by inserting the syringe just below the lateral line

near the tail and was directly smeared on glass slides and air-dried. In case of the clams, the valves were pried open slightly and the needle of a syringe was inserted into the posterior adductor muscle and withdrawn to collect the hemolymph. The smears of both blood and haemolymph were fixed in methanol for 10 min, stained with 5% Giemsa for 15 mins and allowed to dry. The frequency of micronuclei (MNi) was recorded by scoring 2,000 cells per fish / bivalve at 1000x magnification, using an Olympus BX53 microscope. MNi were identified according to the following criteria: (1) spherical or ovoid-shaped extra-nuclear bodies in the cytoplasm (2) a diameter of 1/3 - 1/20 of the main nucleus (3) non-refractory bodies (4) colour texture and optical features resembling those of the nucleus, and (5) the bodies completely separated from the main nucleus (plate 2.3 and 2.4).

2.3.6. Alkaline Single Cell Gel Electrophoresis (Comet Assay)

The comet assay was carried out using the procedure outlined by Lee and Steinert (2003). Twenty microlitres of blood / hemolymph, diluted appropriately with phosphate buffer saline (pH 7.4) was embedded in low melting agarose (LMA) on frosted microscopic slides and then placed in a cold lysing solution (2.5M NaCl, 100mM Na_2EDTA, 10Mm Tris, 10% DMSO and 1% Triton-X pH 10) at 4°C, overnight. All the steps were done in dim light to prevent photo-oxidation of DNA. The slides were then placed in electrophoresis buffer (pH 10) for 15-20 min to allow the DNA to unwind. Electrophoresis was then performed under alkaline conditions (pH 10) for 30 min at 300 mA, 25 V. The slides were placed in neutralization buffer (400mM Tris base, pH 7.5) for 5 min. The gel containing DNA was stained with ethidium bromide and examined using a fluorescence microscope (Olympus BX53) with a green filter at 200x magnification. Randomly selected non-overlapping cells were screened and analyzed with the help of the computer software, CASP (Konca *et al.*,

2003) and the % tail DNA was recorded (Fig 3A and B). The % tail DNA is the amount of DNA (in percent) present in the tail of the "comet" and is used as a measure of DNA damage (Kumaravel and Jha, 2006) (Plate 2.5).

2.3.7. Total Petroleum Hydrocarbon (TPH) analysis

One gram of tissue (muscle, gill and liver of fish and foot and gill of bivalves) was homogenized with 5 ml of hexane in a Potter-Elvehjem homogenizer. The samples were dried by passing through a column of anhydrous sodium sulphate and made to a final volume of 25 ml with hexane. The concentration of total petroleum hydrocarbons was analyzed spectrofluorometrically (Shimadzu RF-5301PC) with excitation at 310 nm and emission at 360 nm. The values were expressed as µg/g of wet tissue (Ansari *et al.*, 2012).

2.3.8. Trace metal analysis

Briefly, tissue samples (0.1 gm) were digested with 10 ml of 14M nitric acid, 5 ml of 13M perchloric acid and 5 ml of deionized water in teflon tubes and heated at 150°C for 2 hours. The solution was then allowed to evaporate to 5 ml in a Teflon beaker using a hot plate and then cooled and diluted upto 50 ml using deionized water. Concentrations of iron (Fe), manganese (Mn), copper (Cu), cadmium (Cd) and lead (Pb) were determined using a Flame Atomic Absorption Spectrophotometry (Shimadzu AA-6300). Results were expressed as µg/g of wet tissue (Begum *et al.*, 2013).

2.3.9. Statistical analyses

Statistical analysis was performed using IBM© SPSS 23 statistical software package. The relationship between MN and DNA damage was analyzed employing Pearson's correlation analysis. The data were tested to meet the assumptions of normality using Shapiro-Wilk's

test and homogeneity using Levene's test and were subsequently arcsine transformed to improve linearity. These values were then used for various parametric tests. The statistical significance of the data of micronucleus test and comet assay were analyzed using Student's t-test to test the individual differences between control and pollution exposed groups. A multiple regression model was used to evaluate the factors that may affect DNA damage. To reduce the number of variables that may be inter-correlated, a principle components analysis (PCA) was performed using pollutant concentrations in the environment, pollutant concentrations in the different tissues of the organisms and physico-chemical parameters. The various seasons and sites were converted to dummy variables and also used in the PCA. In order to enhance the interpretation of factor loadings, Varimax rotation with Kaiser Normalization was used and the factor coefficients so obtained were then used to derive factor scores. Factors with eigenvalues greater than one were used as predictors for multiple regression analysis in order to eliminate multicollinearity. The correlation coefficients between DNA damage and tissue concentrations of trace metals across the sites and seasons were compared between tissues using a z-test. The results were regarded as statistically significant at $p < 0.001$, 0.01 and 0.05.

2.4. Results

2.4.1. Micronucleus test

The results of the micronucleus test in the blood cells of finfishes are represented in fig. 2.3. Fish collected from Vasco and Dona Paula showed a significantly high incidence of micronuclei ($p < 0.05$), whereas those collected from Miramar were insignificant compared to those of Palolem. The highest % MN (0.66 ± 0.09, $p < 0.05$) was recorded in the blood cells of *Arius arius* collected from Vasco in the monsoon season of 2012. In all the seasons

from June 2012 to May 2014, the % of MN were found to be significant in the blood cells of *Johnius dussumieri* collected from Vasco and Dona Paula. A similar observation was noted for *Arius arius* except during the pre-monsoon season from Dona Paula.

The % of MN induced in the hemocytes of bivalves are given in fig. 2.4. Significant % MN was observed in *Meretrix casta* ($p < 0.05$) collected in all the seasons from Vasco compared to those to those of Palolem. Similarly, the % of MN was also found to be significant in *Meretrix ovum* collected from Vasco during all the seasons ($p < 0.05$).

2.4.2. Comet assay

The DNA damage observed in the form of % tail DNA represented in fig. 2.5 was significantly high in the fish collected from Vasco and Dona Paula ($p < 0.05$, 0.01) compared to those of the controls collected from Palolem. Significant DNA damage ($p < 0.05$) was also observed in the fish collected from Miramar. The highest % tail DNA was induced in the blood cells of *Arius arius* from Vasco (19.42 ± 1.06, $p < 0.01$) in the post-monsoon season of 2012-2013. DNA damage was significant in *Johnius dussumieri* and *Arius arius* collected from all the sites and all the seasons except from Dona Paula during the pre-monsoon season and Miramar during the same season.

The % tail DNA observed in the hemocytes of the bivalves is represented in fig. 2.6. DNA damage as observed by the comet assay was found to be significant in both *Meretrix casta* and *Meretrix ovum* collected from Vasco in all the seasons ($p < 0.05$) compared to those collected from Palolem.

The pooled data of the MN test and comet assay from both finfish and shellfish were further analysed using Pearson's correlation analysis (fig. 2.7). The % MN and % tail DNA were found to be significantly positively correlated with one another ($R^2 = 0.80$, $p < 0.01$).

2.4.3. Total petroleum hydrocarbons

TPH concentrations observed in the tissues of finfishes are represented in fig. 2.8-2.10. In general, significant concentrations of TPHs were observed in all the tissues (muscle, gill and liver) of *J. dussumieri* and *A. arius* from Vasco, Dona Paula and Miramar compared to those from Palolem. Further, in both fish at these sites, the TPH concentrations in liver were higher than those in the gill and muscle tissues. The highest concentration of TPHs was recorded in the liver of *A. arius* (7.74 ± 0.1 µg/g, $p < 0.01$) from Vasco during the pre-monsoon season in 2013.

The concentration range of TPHs in the tissues (foot and gill) of shellfish represented in fig. 2.11, were much higher compared to those in finfishes. Significantly high concentrations of TPH were recorded for both *M. casta* and *M. ovum* collected from Vasco compared to those from Palolem in all the seasons. Highest concentrations were recorded in the gill tissues of both *M. casta* (29.36 ± 0.11, $p < 0.001$) and *M. ovum* (28.72 ± 0.09, $p < 0.001$) collected from Vasco during the pre-monsoon season in 2013.

2.4.4. Trace metals

Trace metal concentrations observed in the tissues of finfishes are represented in figs. 2.12 - 2.26. High concentrations of Fe, Mn, Cu, Cd and Pb were found in the muscle, gills and livers of both *J. dussumieri* and *A. arius*. Comparatively, Fe, Cd and Pb concentrations were found to be consistently high in the tissues of these fish collected from Vasco, Dona Paula

and Miramar in all the seasons compared to those of Palolem. The concentrations of Fe in the tissues of both the fish were found to be highly significant in Vasco and Dona Paula in all the seasons during the study period. The concentration of Cu was found to be the least in the tissues compared to the other metals.

The concentrations of trace metals in the tissues of *M. casta* and *M. ovum* are given in figs 2.27 -2.31. The concentration of Fe was found to be more prevalent in the gills than the foot in both the bivalves collected from Vasco. However, Mn was found to be significant in both the foot and gill tissues in both species collected from Vasco during all seasons. The concentration of Cd was found to be highly significant in the bivalves from Vasco compared to the range of values at Palolem. Cu and Pb were also found in significant quantities in the tissues of the bivalves from Vasco compared to Palolem.

Based on the pooled data from finfish and shellfish (Table 1), the DNA damage was found to be significantly correlated by the concentrations of TPH, Fe, Mn, Cu, Cd and Pb in the tissues of finfish whereas the induction of DNA damage in case of bivalves were dependent on the concentrations of TPHs, Fe, Cu, Cd and Pb.

2.4.5. Principal components analysis

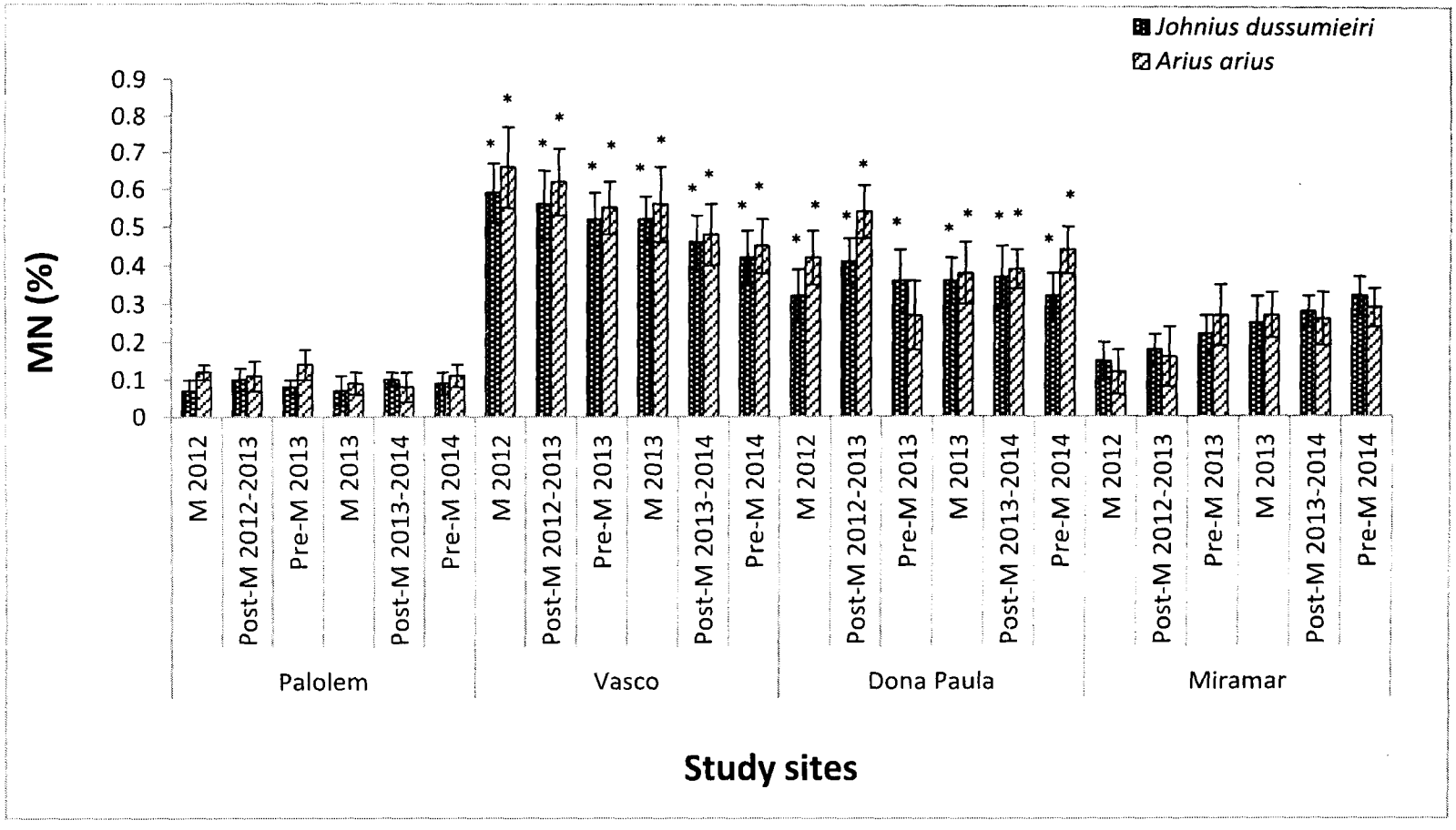
Ten components were extracted from the principal component analysis after varimax rotation of the factor axes which accounted for 87.22% of the total variance of the original variables (figs. 2.32 and 2.33). The first component which had a variance of 52.24% was characterized by high positive loadings of tissue and environmental concentrations of pollutants. The second component which explained a variance of 8.24% was characterized by loadings of

physico-chemical parameters of surface and bottom water. The variables that were associated with the remaining components were the different seasons.

2.4.6. Multiple regression analysis

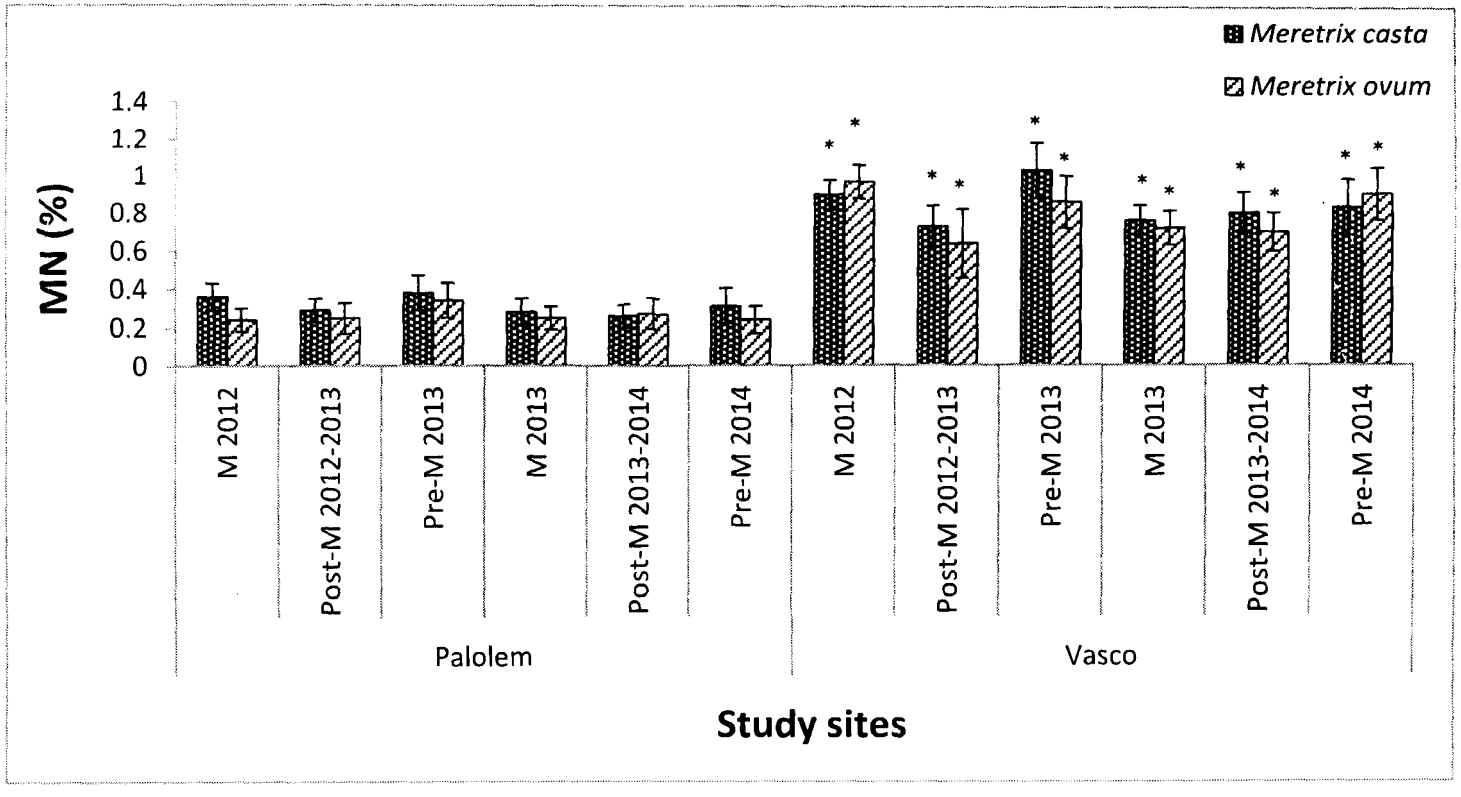
After factor scores were obtained from the ten components, the score values were used as independent variables and DNA damage (% tail DNA) was used as the dependent variable due to the high correlation between the comet assay and micronucleus test in the present study (table 2.2). The regressions of standardized DNA damage on component 1 was the most significant ($p < 0.0001$).

Fig. 2.3. Micronuclei (%) in cells of finfish (Mean ± SD) collected from various study sites in different seasons from June 2012 to May 2014



Note: M = monsoons, * $p < 0.05$

Fig. 2.4. Micronuclei (%) in cells of shellfish (Mean ± SD) collected from various study sites in different seasons from June 2012 to May 2014



Note: M = monsoons, * $p < 0.05$

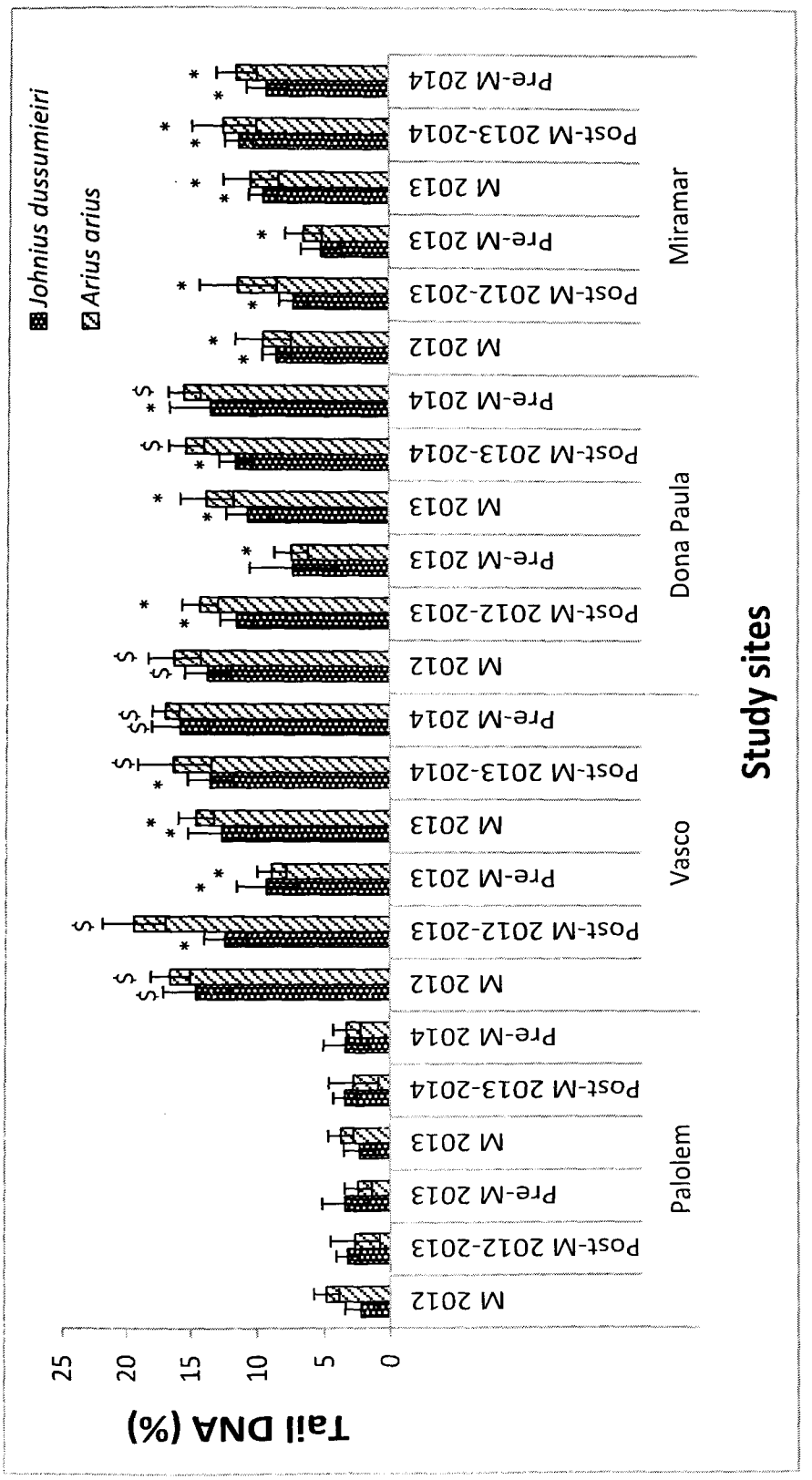
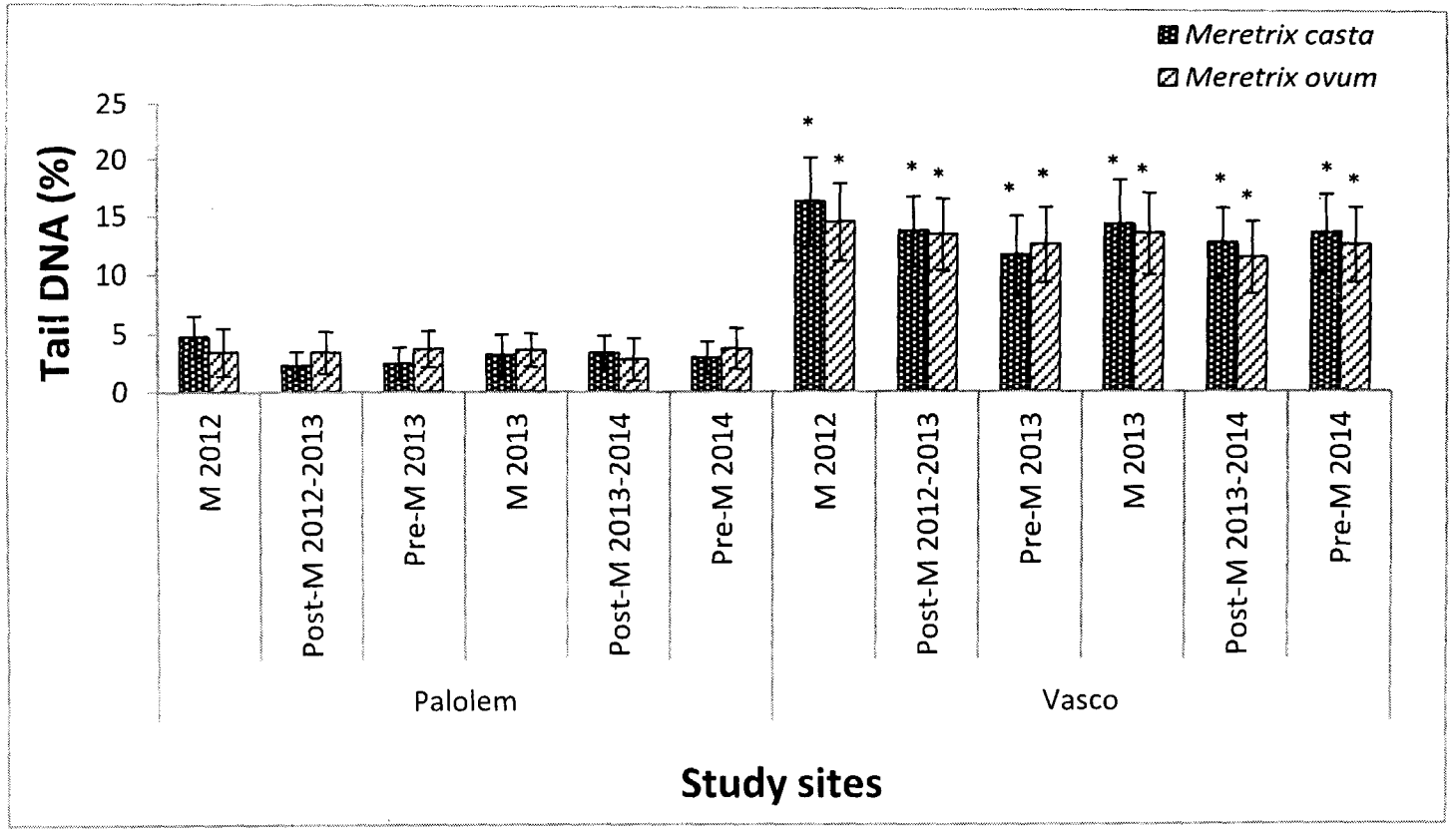


Fig. 2.5. Tail DNA (%) in cells of finfishes (Mean ± SD) collected from various study sites in different seasons from June 2012 to May 2014

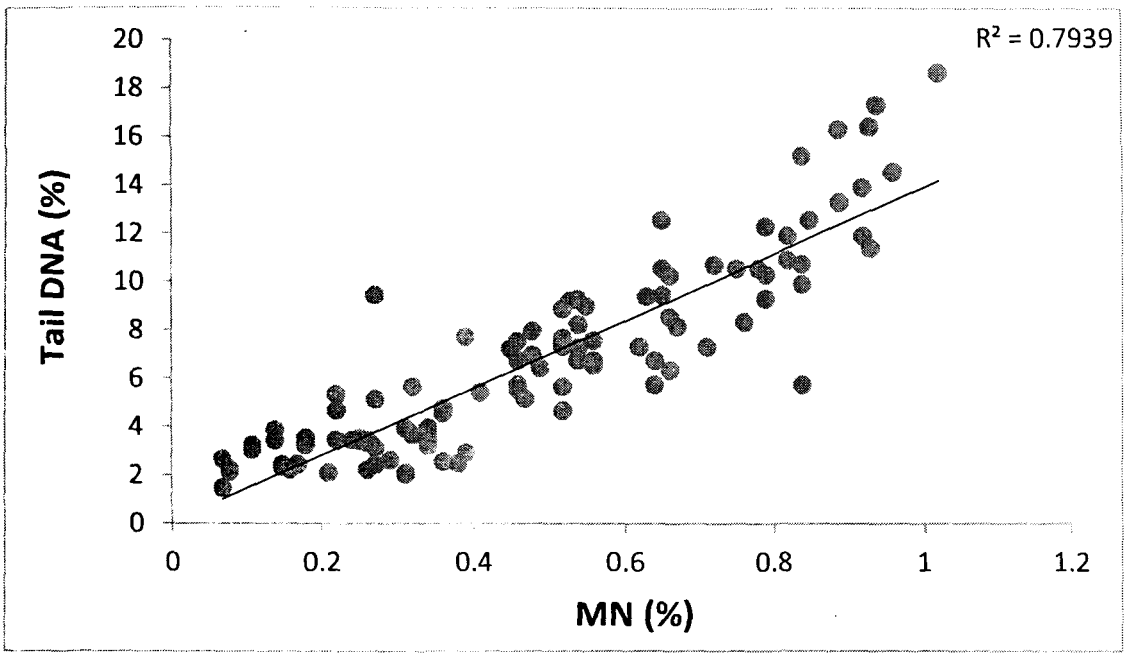
Note: M = monsoons, $ $p < 0.05$, * $p < 0.05$

Fig. 2.6. Tail DNA (%) in cells of shellfish (Mean ± SD) collected from various study sites in different seasons from June 2012 to May 2014



Note: M = monsoons, * $p < 0.05$

Fig. 2.7. Correlation analysis between MN (%) and Tail DNA (%) of pooled data from finfish and shellfish



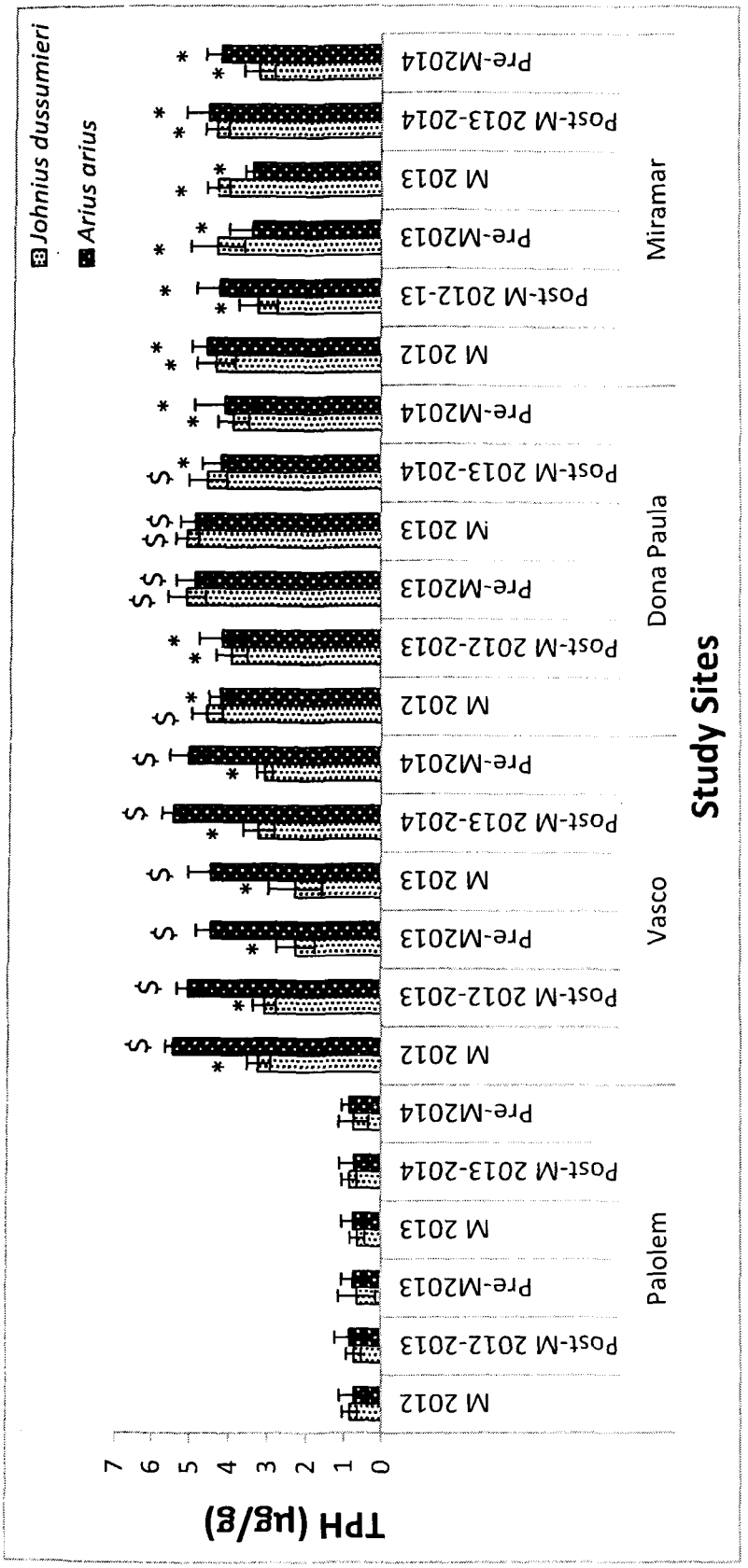


Fig. 2.8. TPH (Mean ± SD) in muscle tissue of finfishes collected from various study sites in different seasons from June 2012 to May 2014

Note: M = monsoons, $ $p < 0.01$, * $p < 0.05$

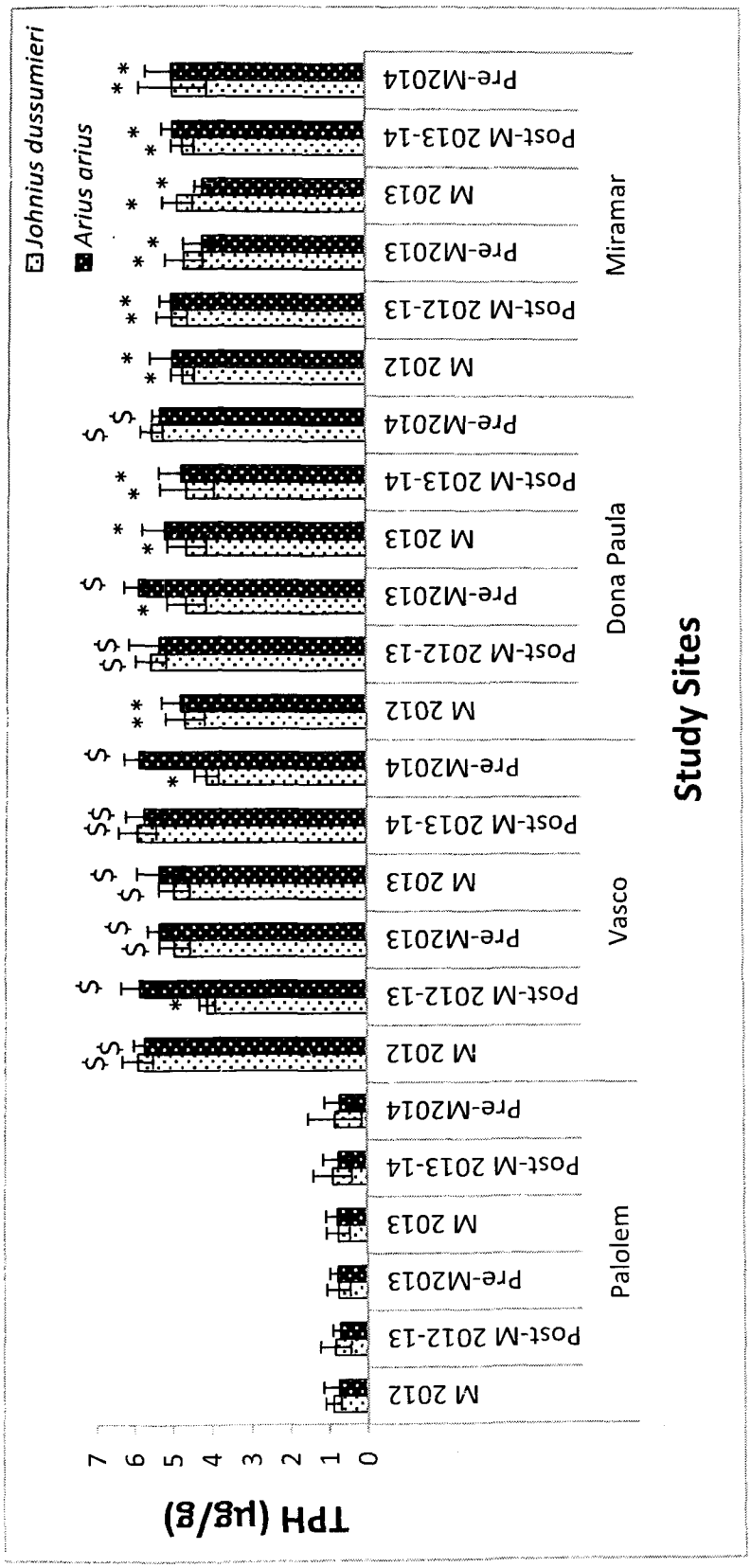


Fig. 2.9. *TPH (Mean ± SD) in gill tissue of finfishes collected from various study sites in different seasons from June 2012 to May 2014*

Note: M = monsoons, $ $p < 0.01$, * $p < 0.05$

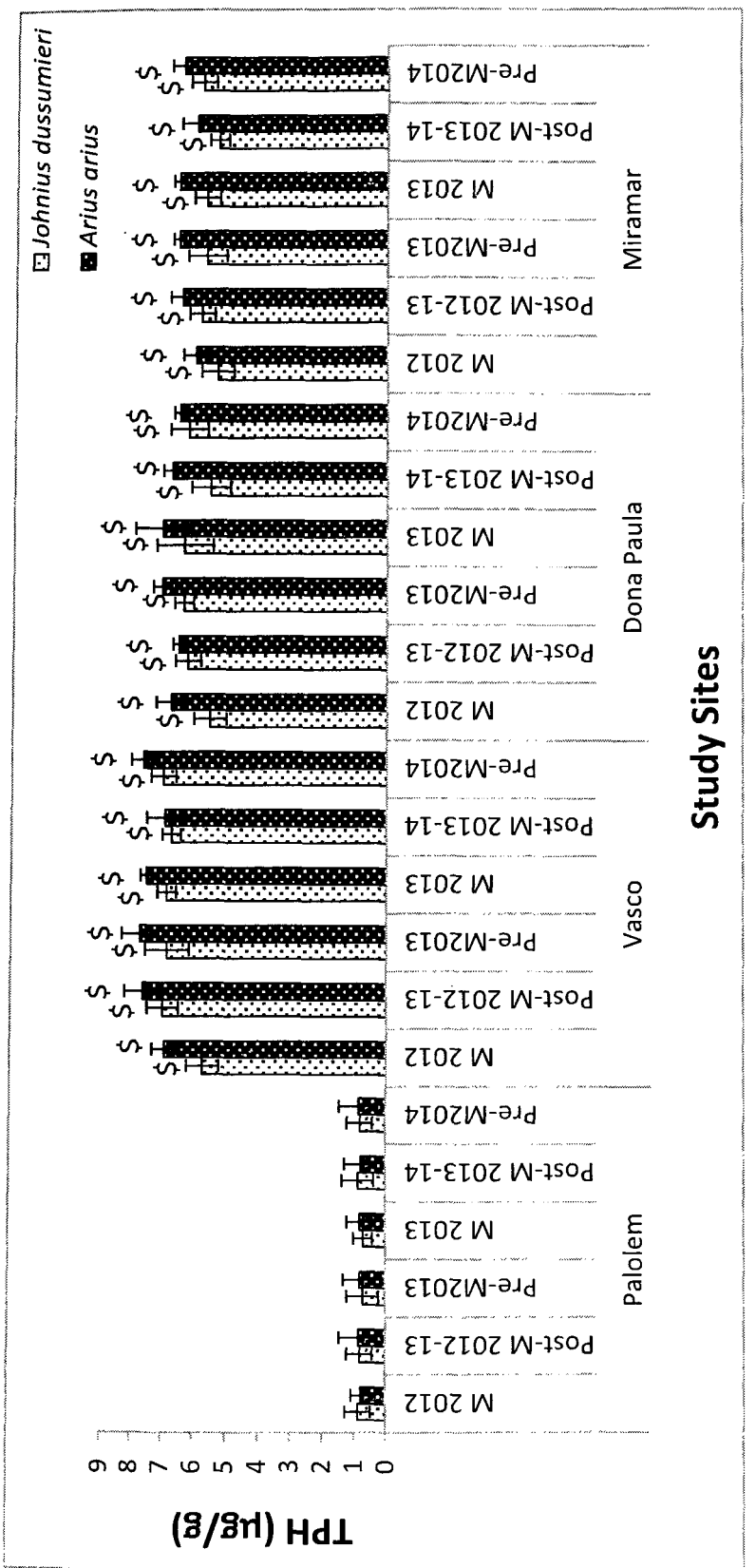
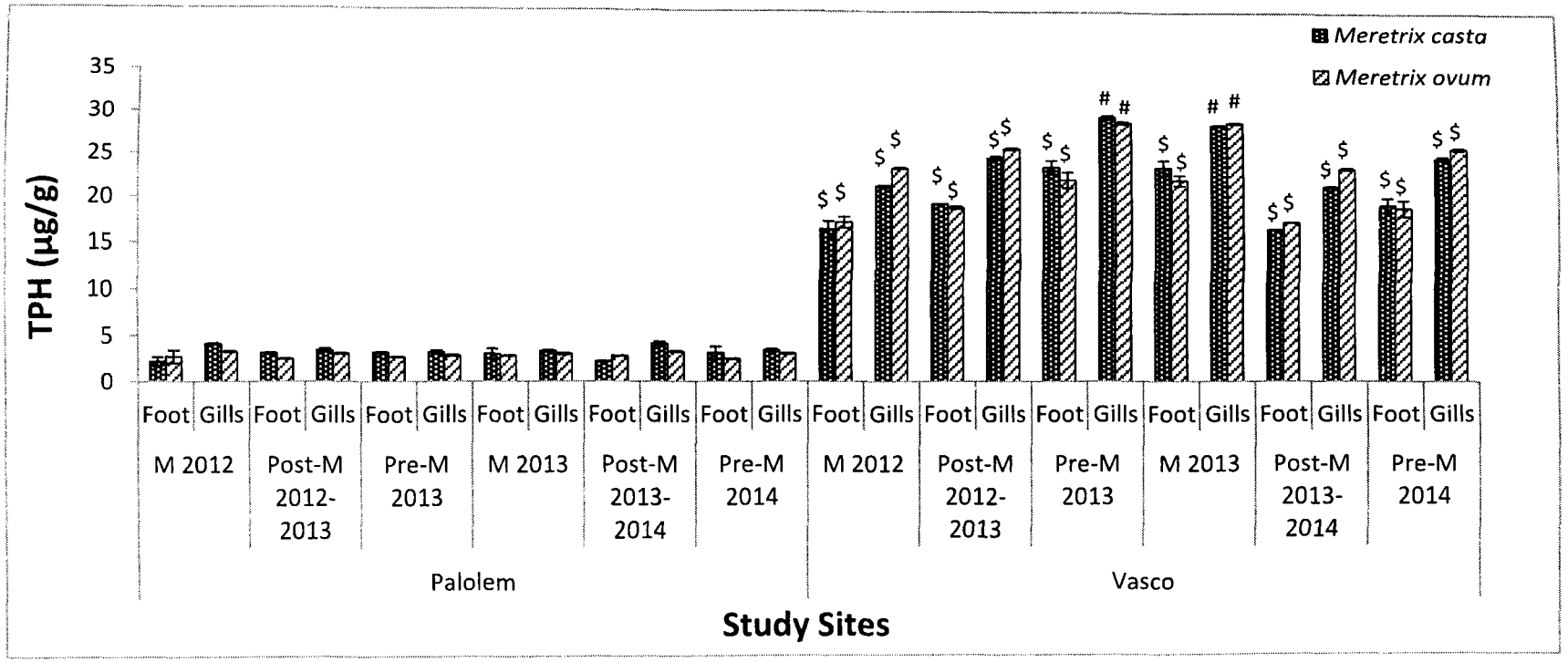


Fig. 2.10. *TPH (Mean ± SD) in liver tissue of finfishes collected from various study sites in different seasons from June 2012 to May 2014*

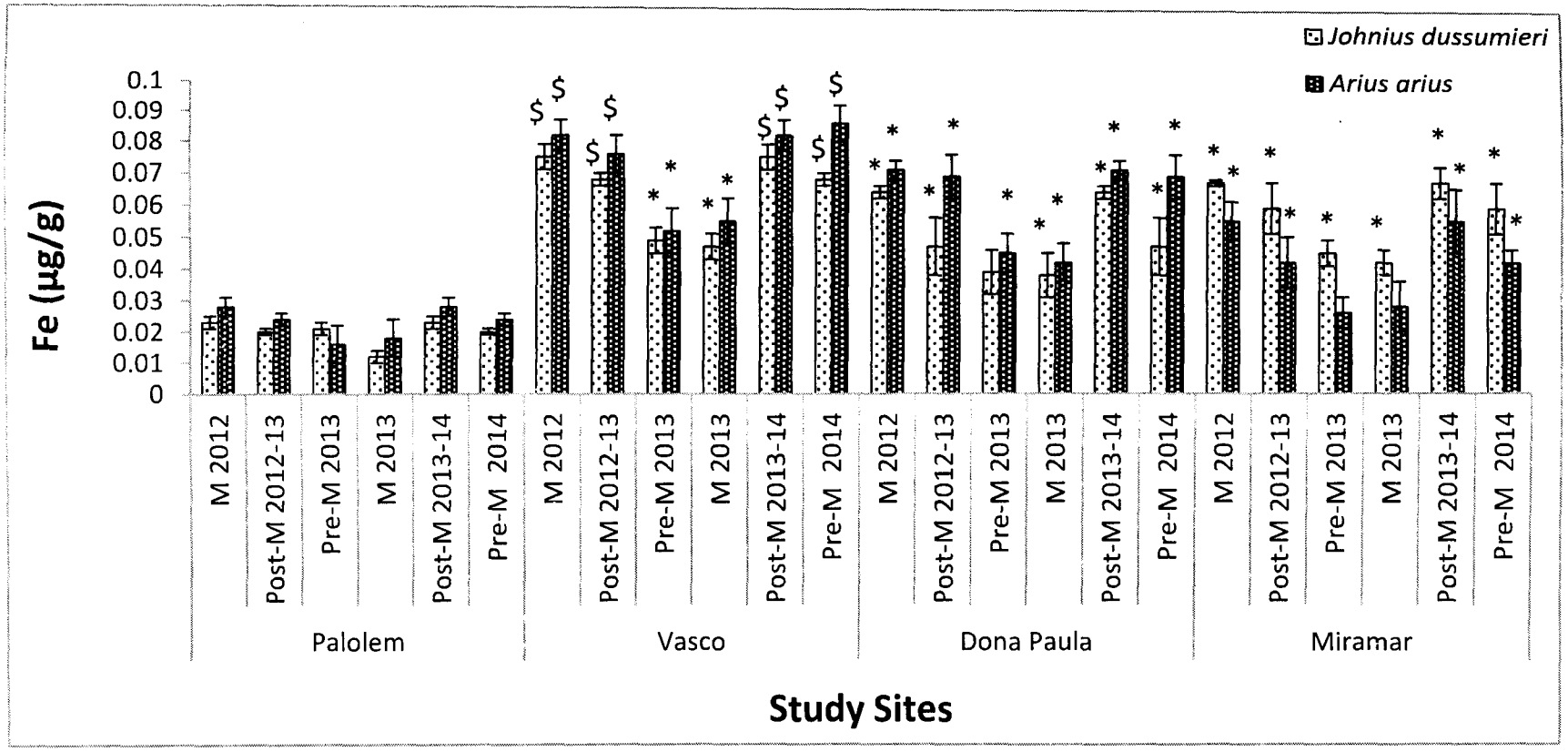
Note: M = monsoons, $ $p < 0.01$

Fig. 2.11. TPH (Mean ± SD) in tissues of shellfish collected from various study sites in different seasons from June 2012 to May 2014



Note: M = monsoons, # $p < 0.001$, $ $p < 0.01$

Fig. 2.12. Fe (Mean ± SD) in muscle tissue of finfishes collected from various study sites in different seasons from June 2012 to May 2014



Note: M = monsoons, $ $p < 0.01$, * $p < 0.05$

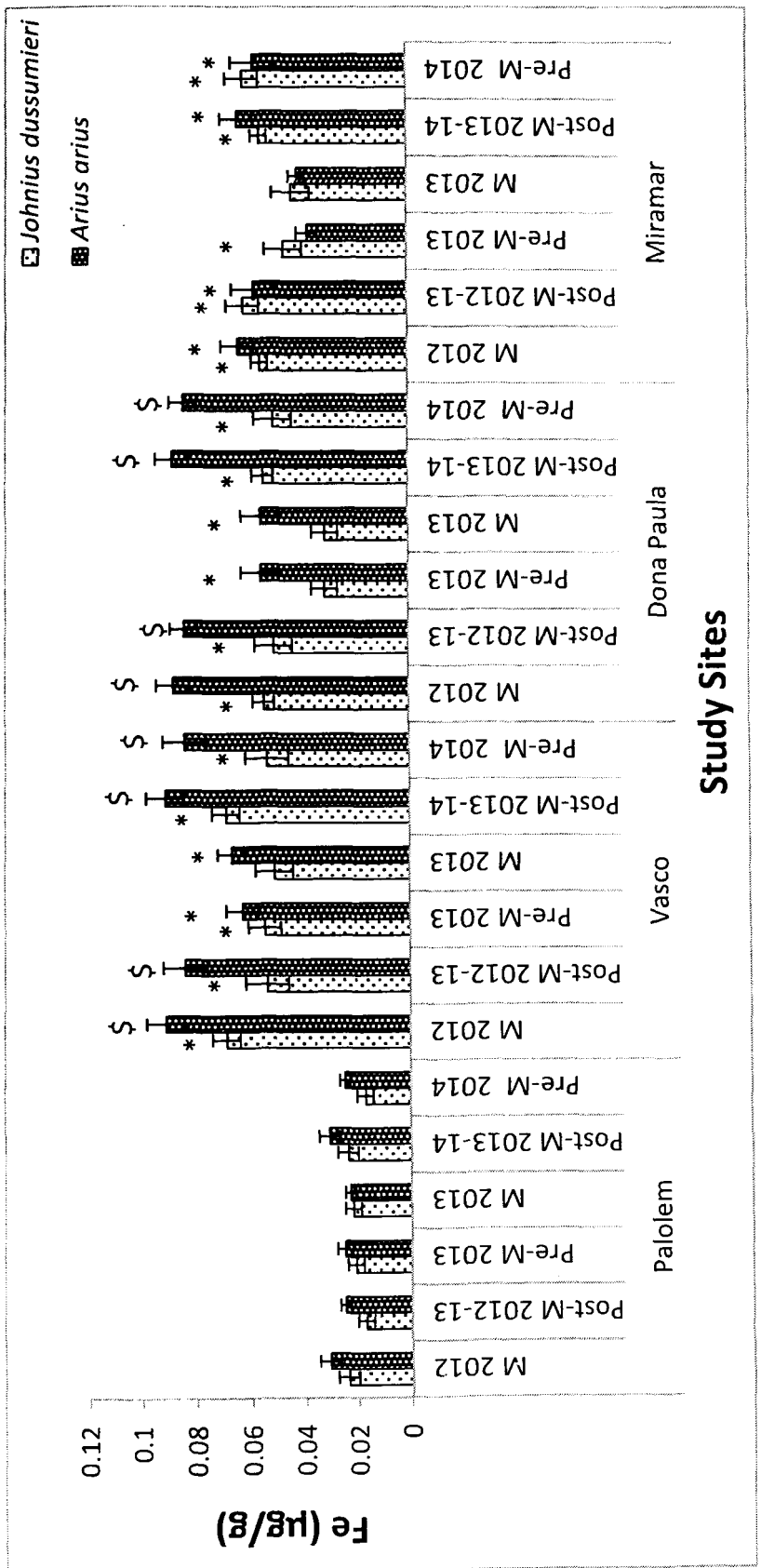
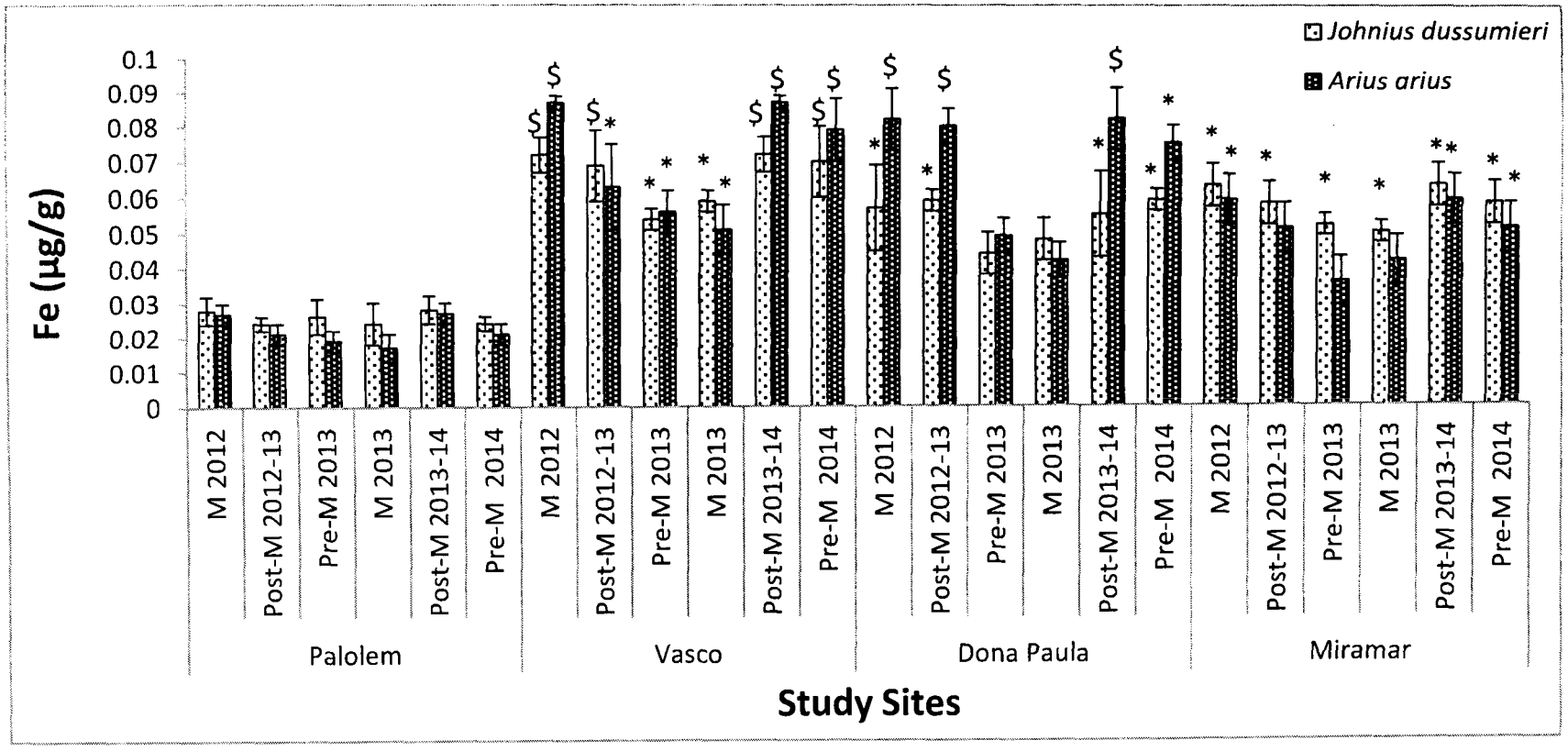


Fig. 2.13. Fe (Mean ± SD) in gill tissue of finfishes collected from various study sites in different seasons from June 2012 to May 2014

Note: M = monsoons, $ $p < 0.01$, * $p < 0.05$

Fig. 2.14. Fe (Mean ± SD) in liver tissue of finfishes collected from various study sites in different seasons from June 2012 to May 2014



Note: M = monsoons, $ $p < 0.01$, * $p < 0.05$

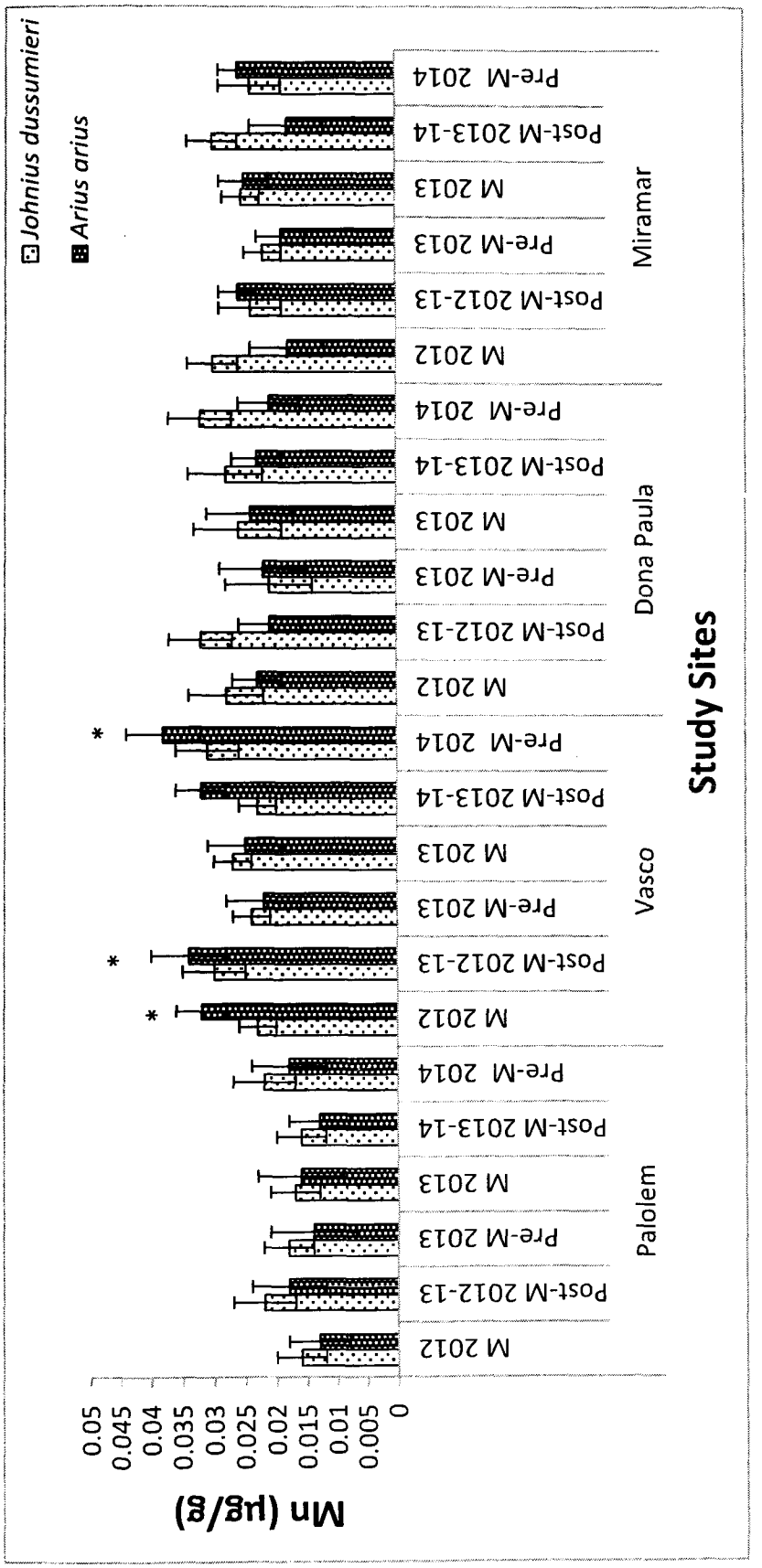


Fig. 2.15. Mn (Mean ± SD) in muscle tissue of finfishes collected from various study sites in different seasons from June 2012 to May 2014

Note: M = monsoons, * $p < 0.05$

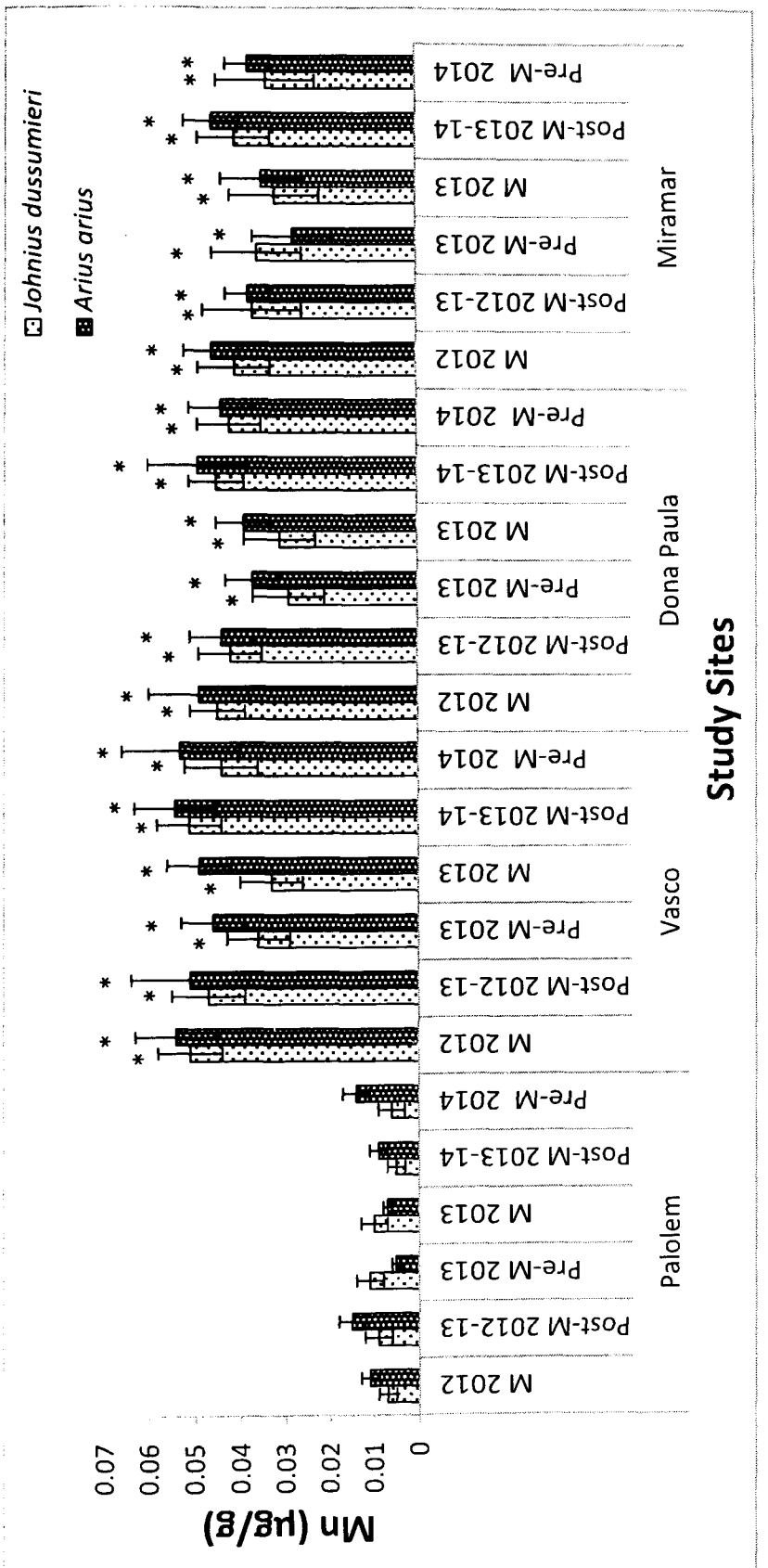


Fig. 2.16. Mn (Mean ± SD) in gill tissue of finfishes collected from various study sites in different seasons from June 2012 to May 2014

Note: M = monsoons, * $p < 0.05$

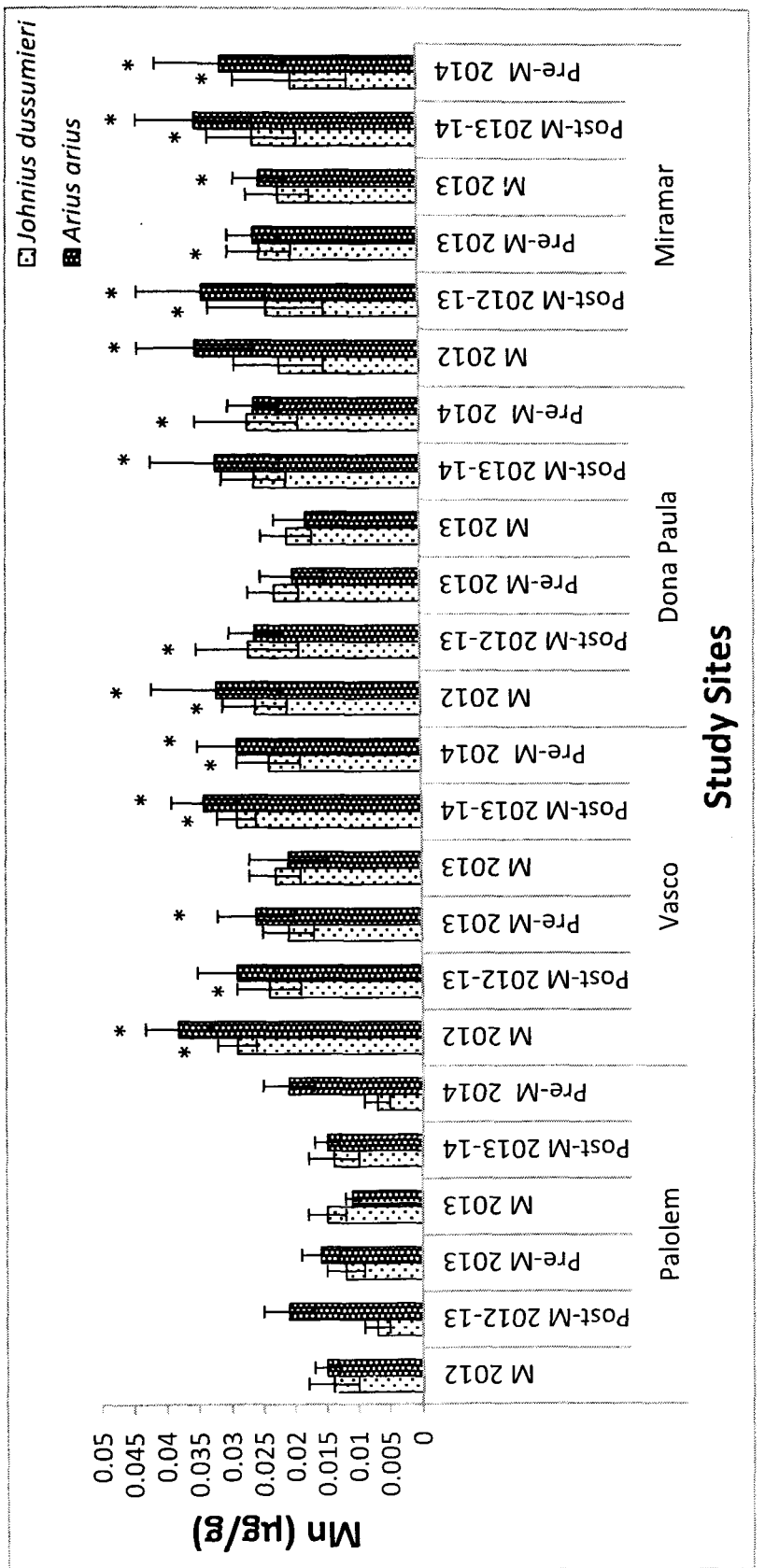


Fig. 2.17. Mn (Mean ± SD) in liver tissue of finfishes collected from various study sites in different seasons from June 2012 to May 2014

Note: M = monsoons, * $p < 0.05$

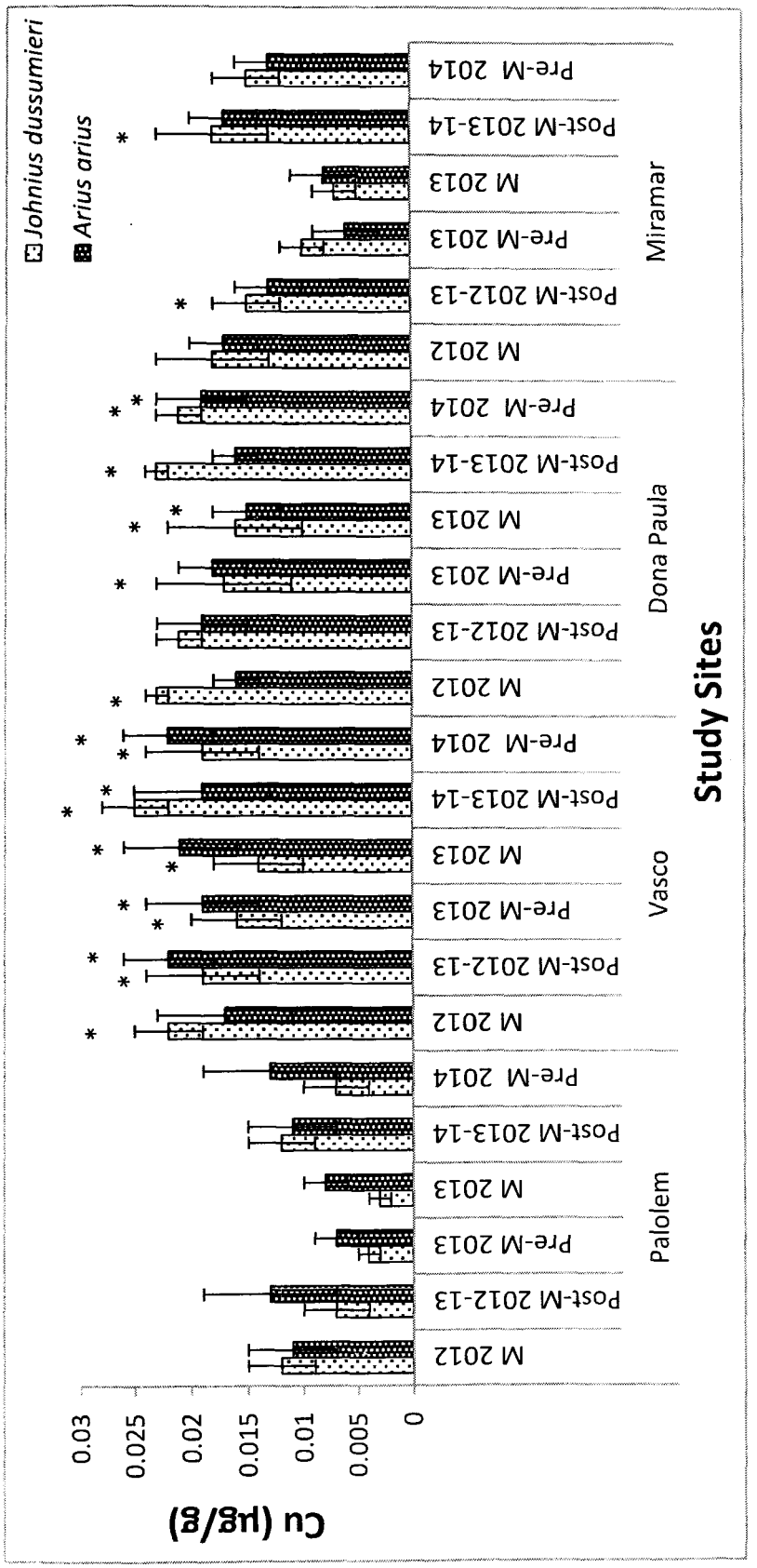


Fig. 2.18. Cu (Mean ± SD) in muscle tissue of finfishes collected from various study sites in different seasons from June 2012 to May 2014

Note: M = monsoons, * $p < 0.05$

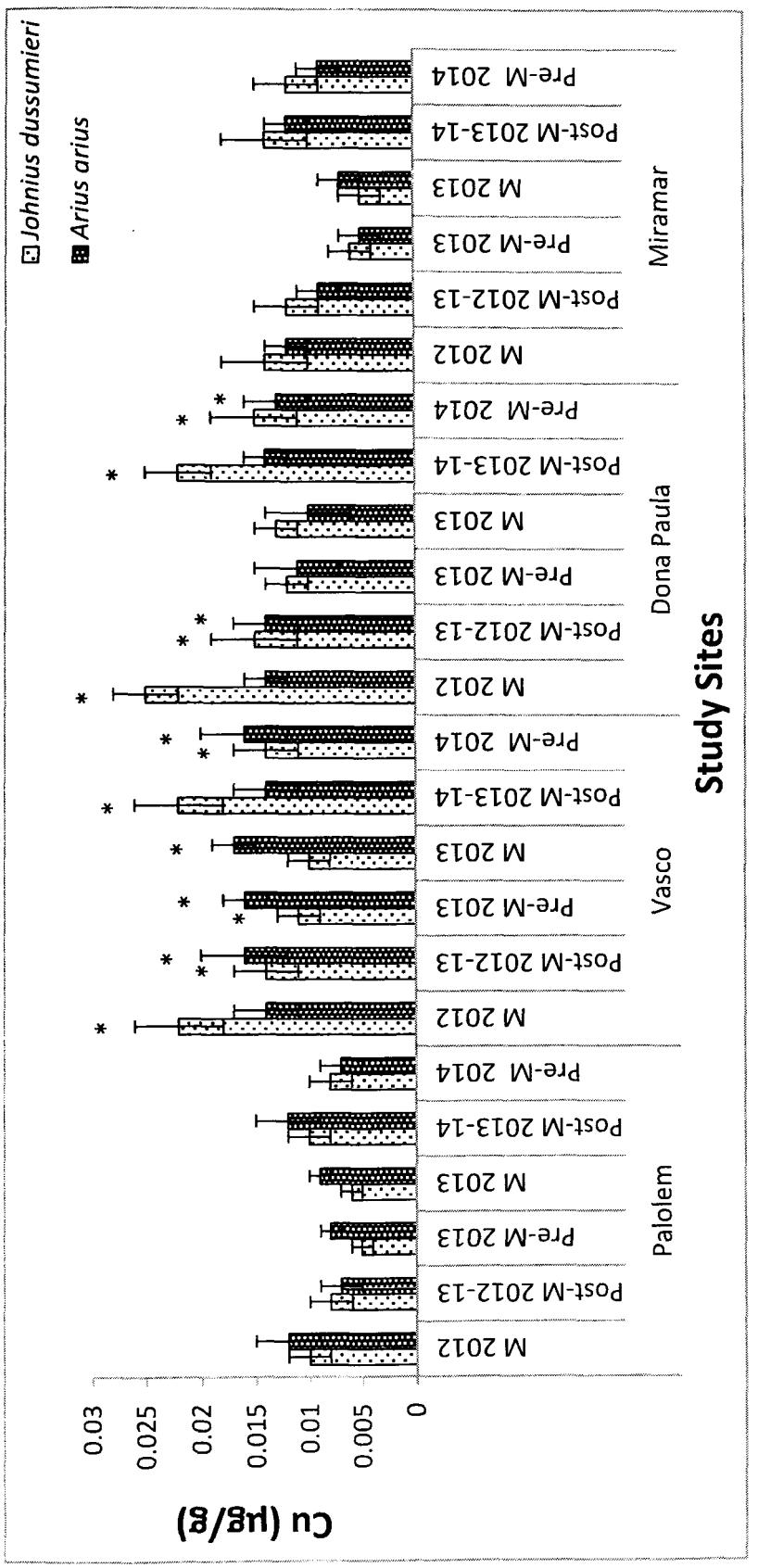
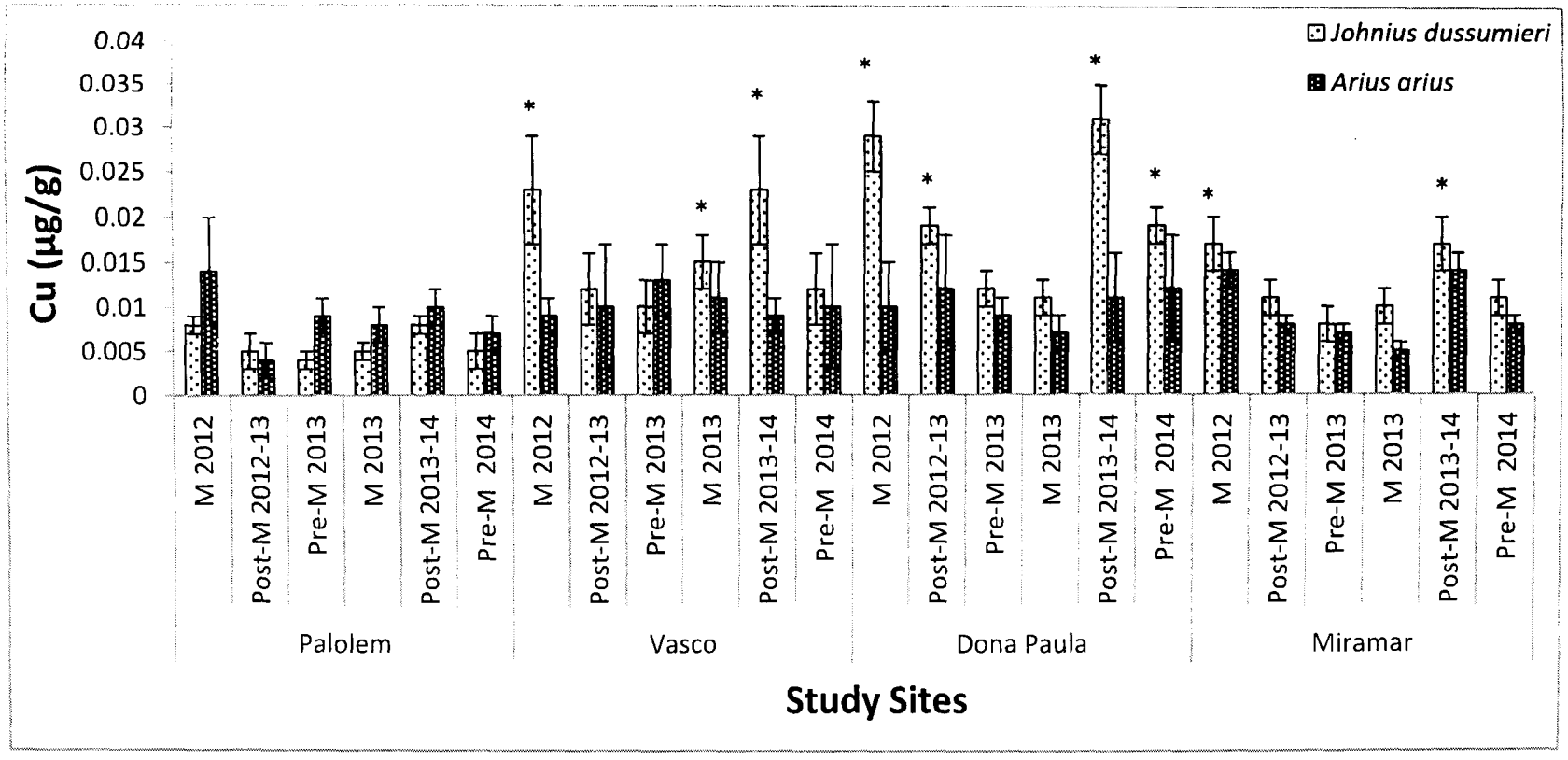


Fig. 2.19. Cu (Mean ± SD) in gill tissue of finfishes collected from various study sites in different seasons from June 2012 to May 2014

Note: M = monsoons, * $p < 0.05$

Fig. 2.20. Cu (Mean ± SD) in liver tissue of finfishes collected from various study sites in different seasons from June 2012 to May 2014



Note: M = monsoons, * $p < 0.05$

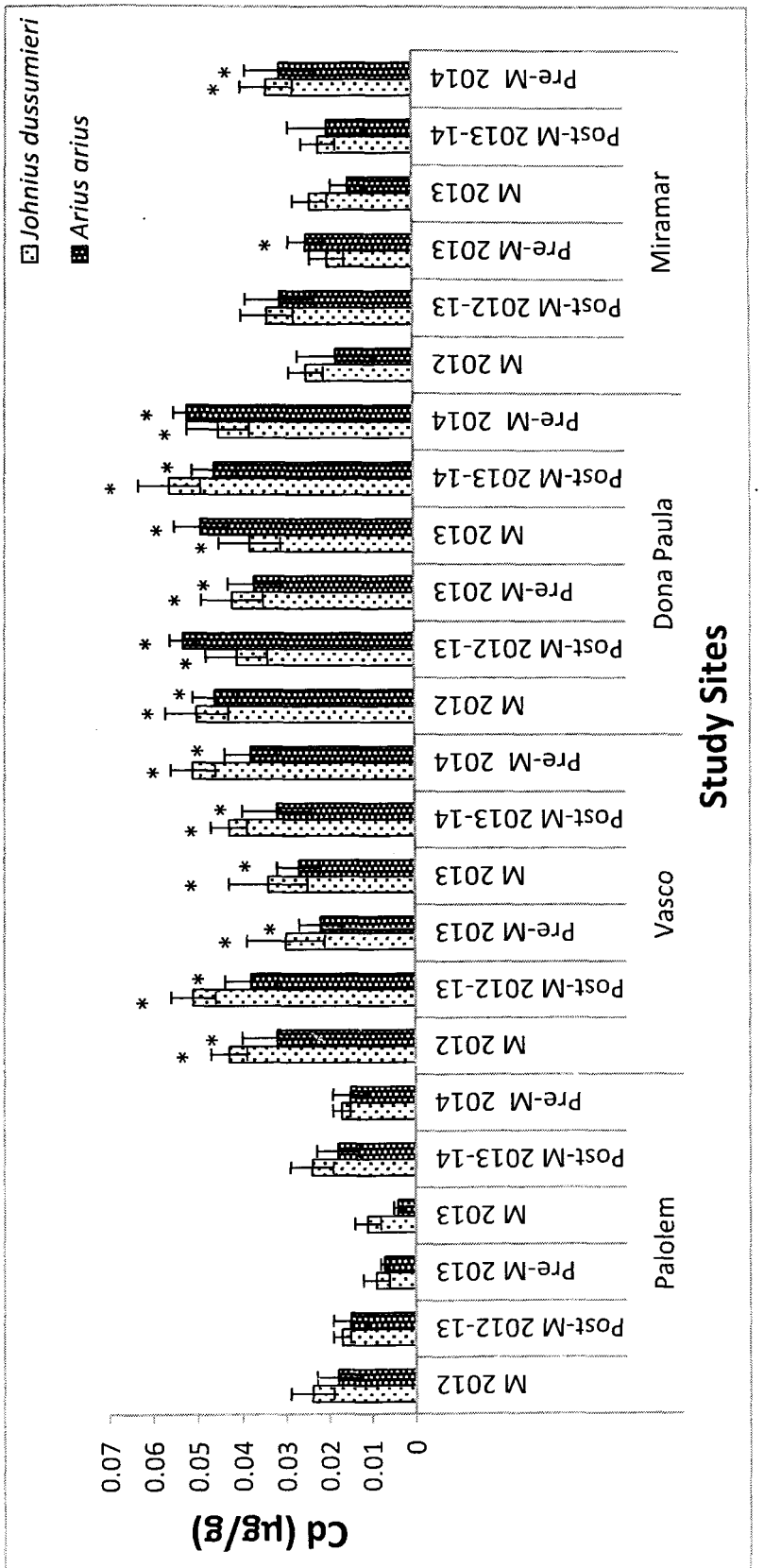


Fig. 2.21. Cd (Mean ± SD) in muscle tissue of finfishes collected from various study sites in different seasons from June 2012 to May 2014

Note: M = monsoons, * $p < 0.05$

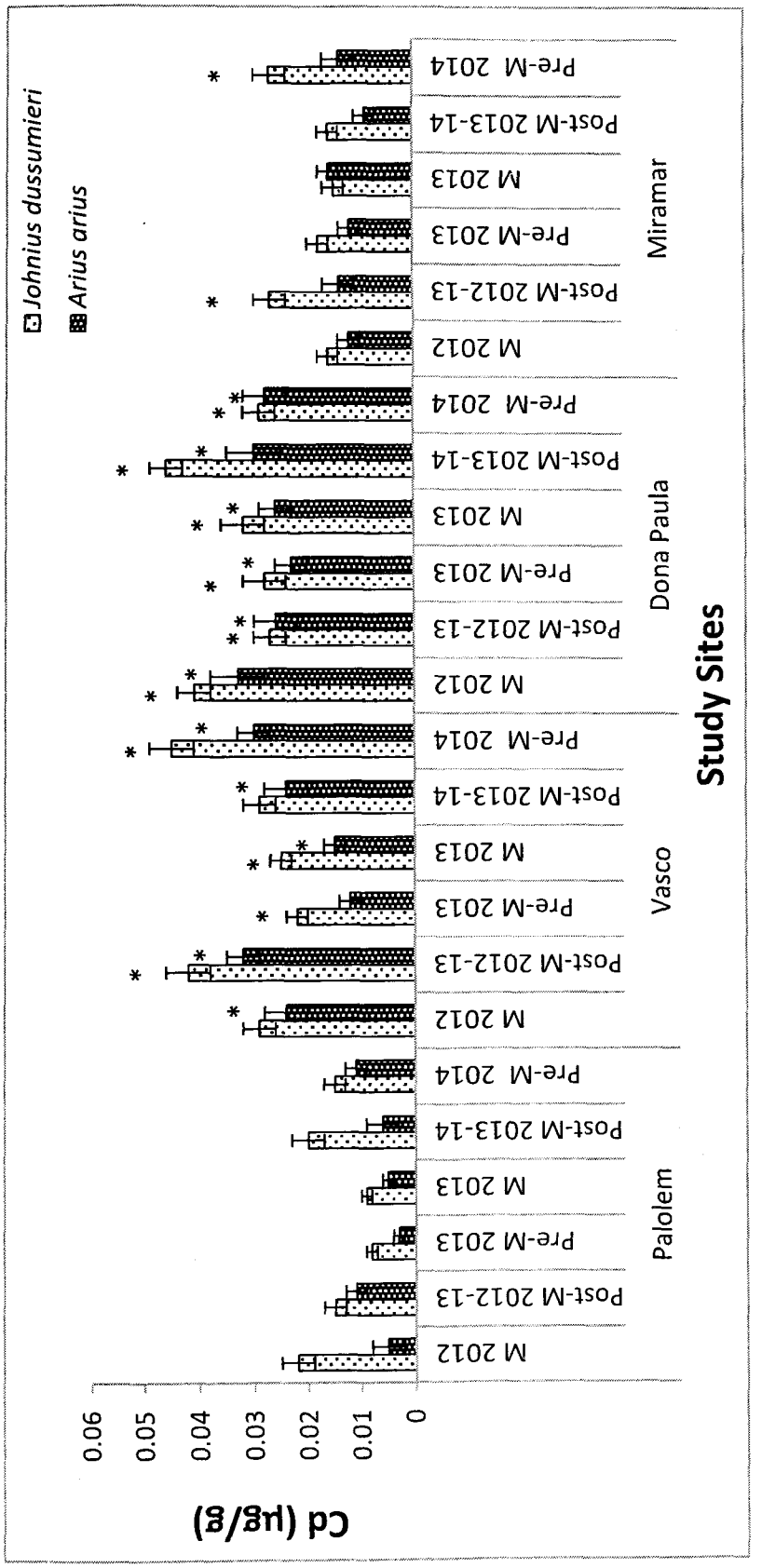
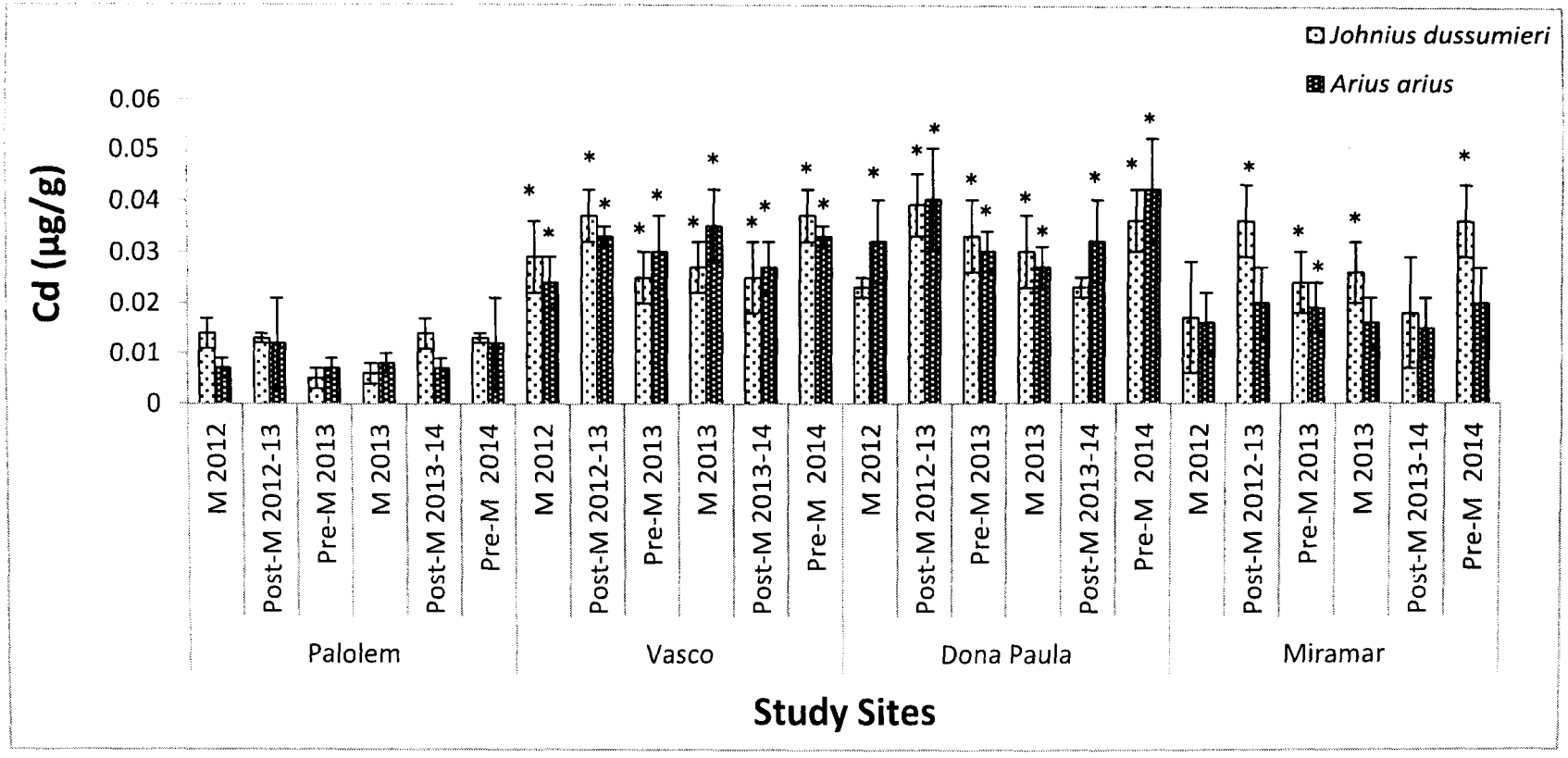


Fig. 2.22. Cd (Mean ± SD) in gill tissue of finfishes collected from various study sites in different seasons from June 2012 to May 2014

Note: M = monsoons, * $p < 0.05$

Fig. 2.23. Cd (Mean ± SD) in liver tissue of finfishes collected from various study sites in different seasons from June 2012 to May 2014



Note: M = monsoons, * $p < 0.05$

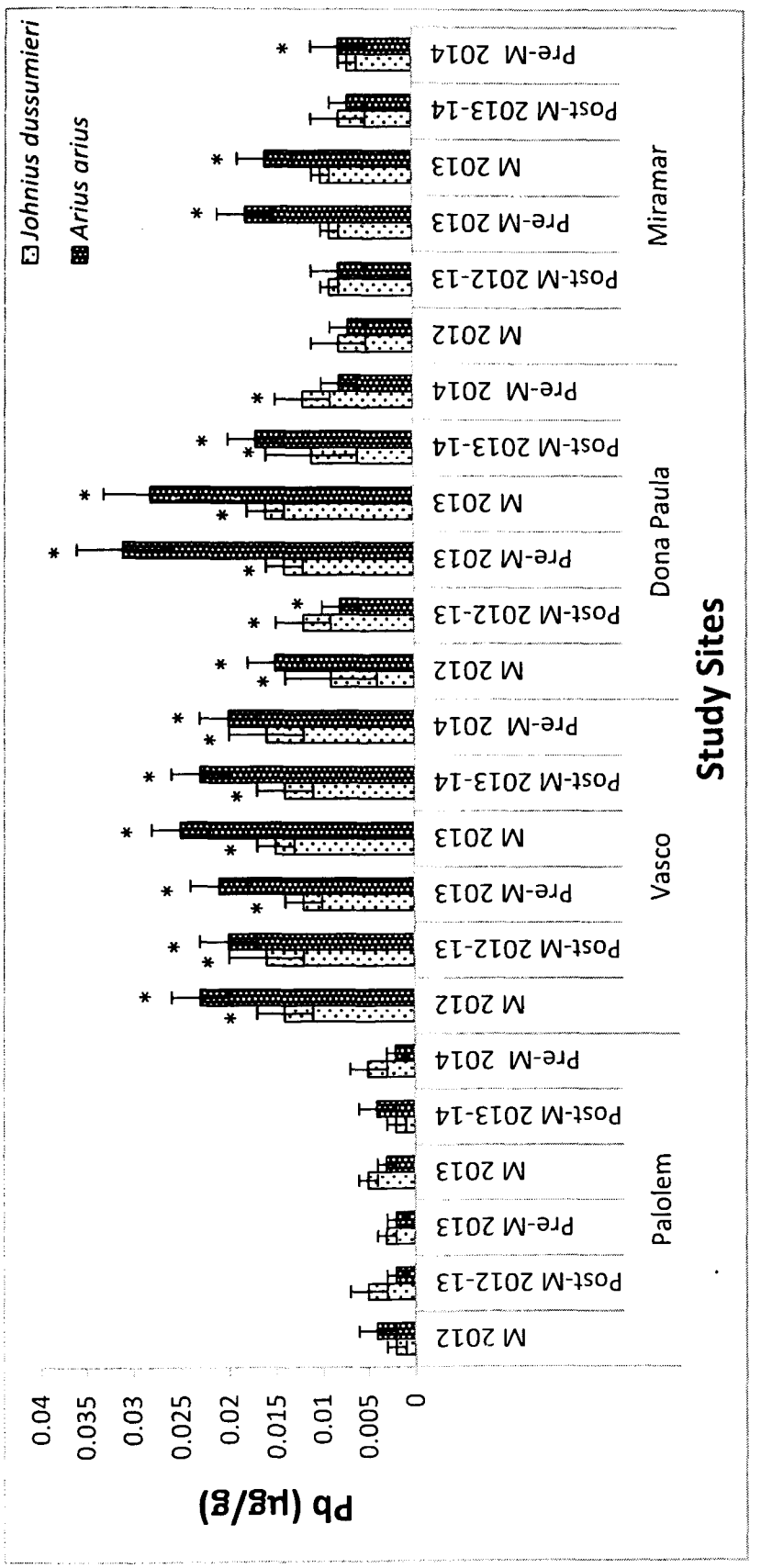


Fig. 2.24. Pb (Mean ± SD) in muscle tissue of finfishes collected from various study sites in different seasons from June 2012 to May 2014

Note: M = monsoons, * $p < 0.05$

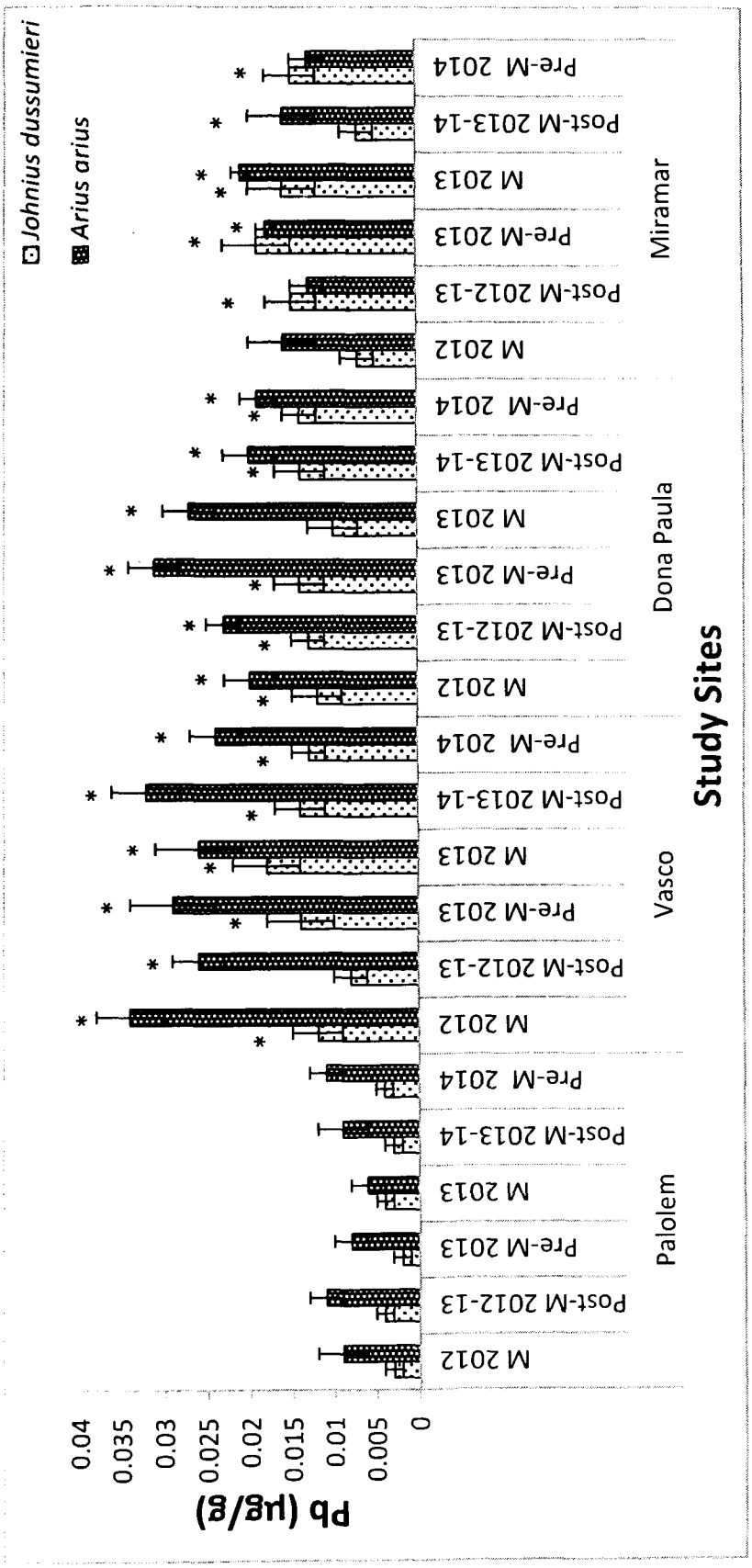


Fig. 2.25. Pb (Mean ± SD) in gill tissue of finfishes collected from various study sites in different seasons from June 2012 to May 2014

Note: M = monsoons, * $p < 0.05$

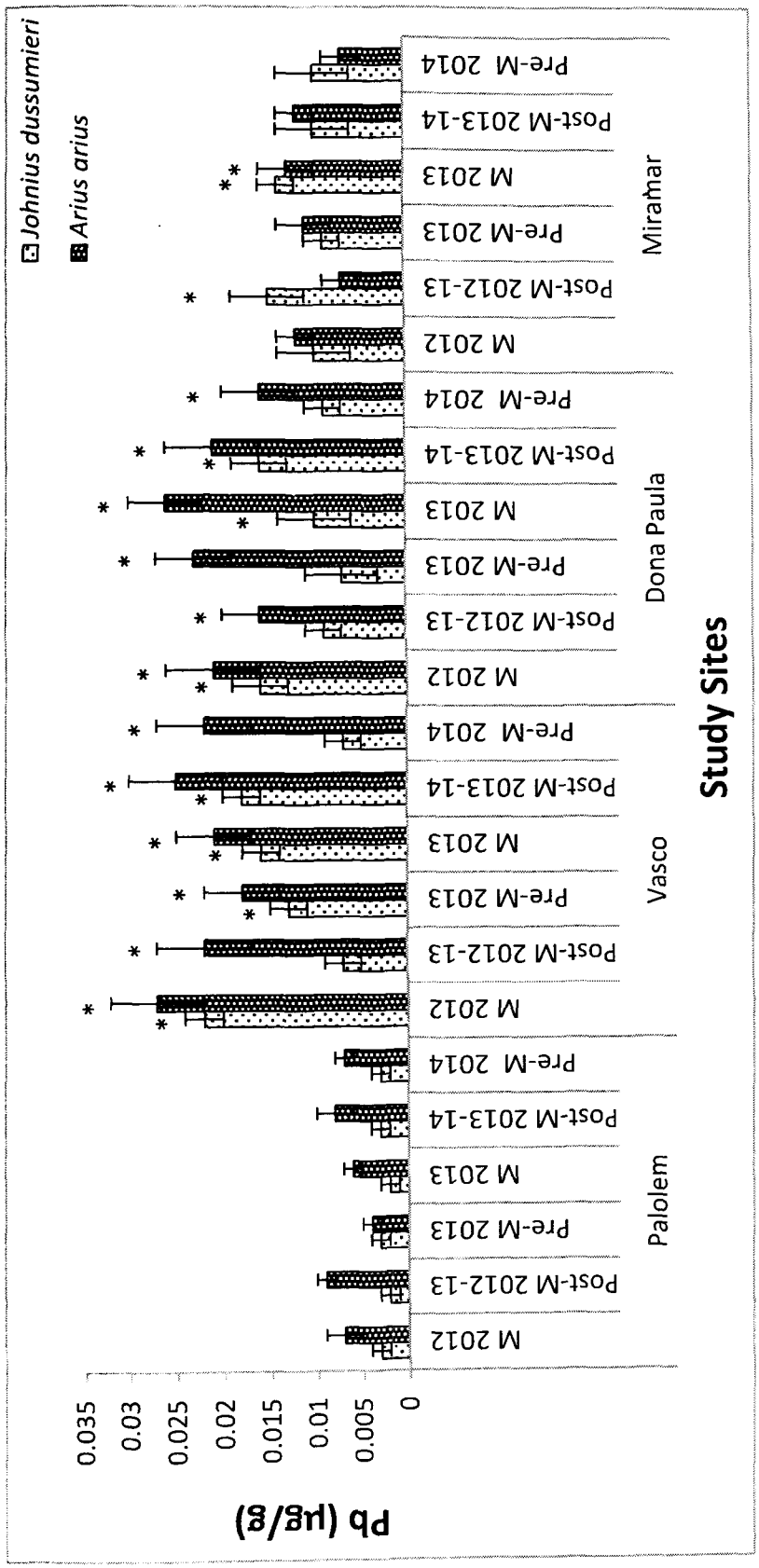
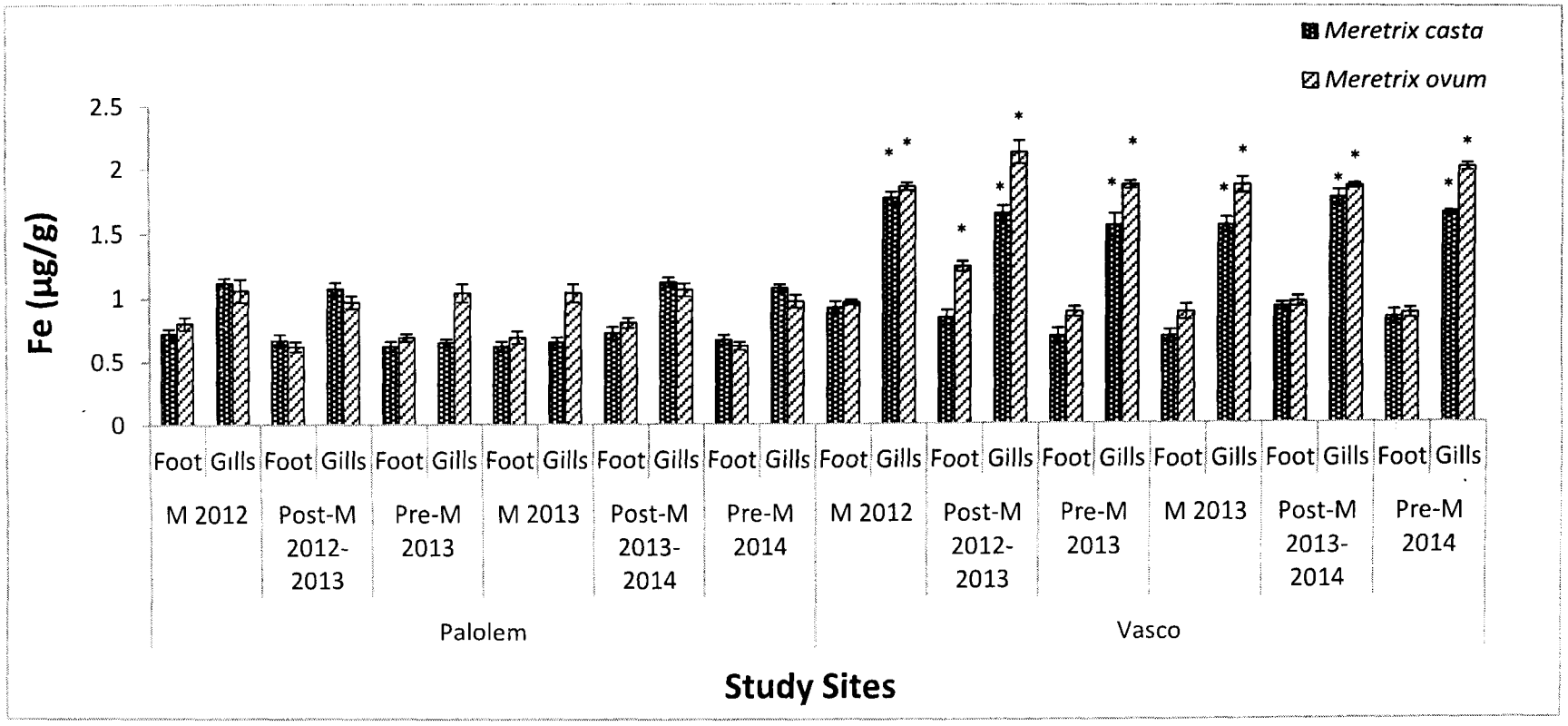


Fig. 2.26. Pb (Mean ± SD) in liver tissue of finfishes collected from various study sites in different seasons from June 2012 to May 2014

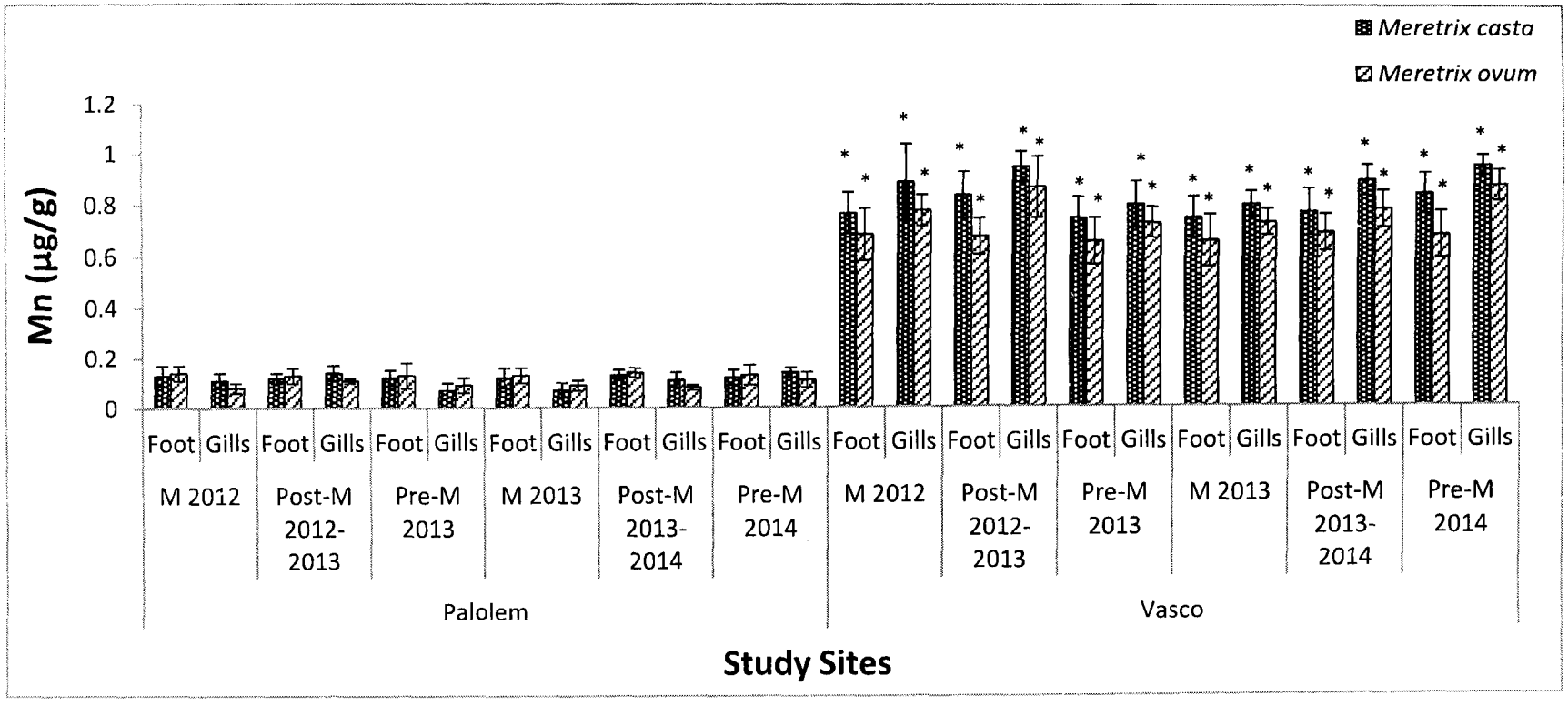
Note: M = monsoons, * $p < 0.05$

Fig. 2.27. Fe (Mean ± SD) in tissues of shellfish collected from various study sites in different seasons from June 2012 to May 2014



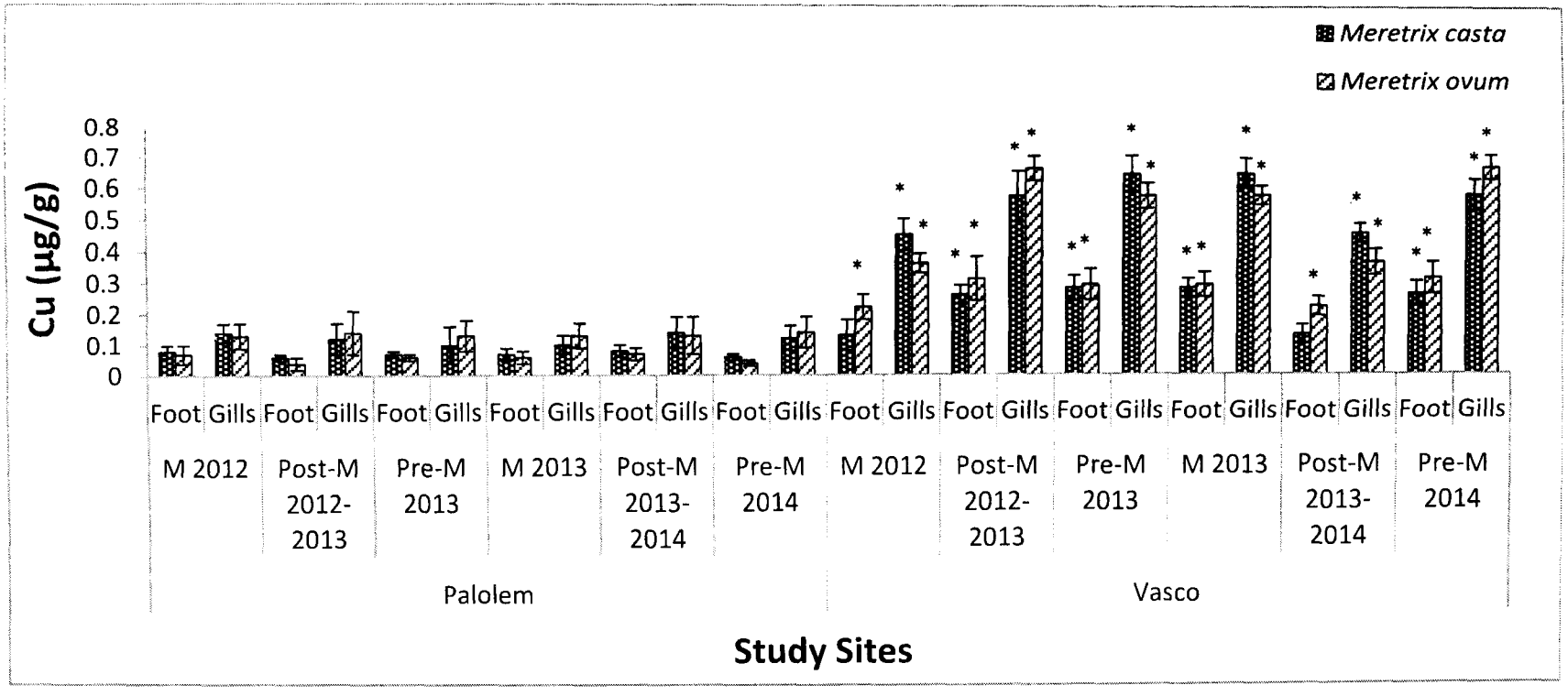
Note: M = monsoons, * $p < 0.05$

Fig. 2.28. Mn (Mean ± SD) in tissues of shellfish collected from various study sites in different seasons from June 2012 to May 2014



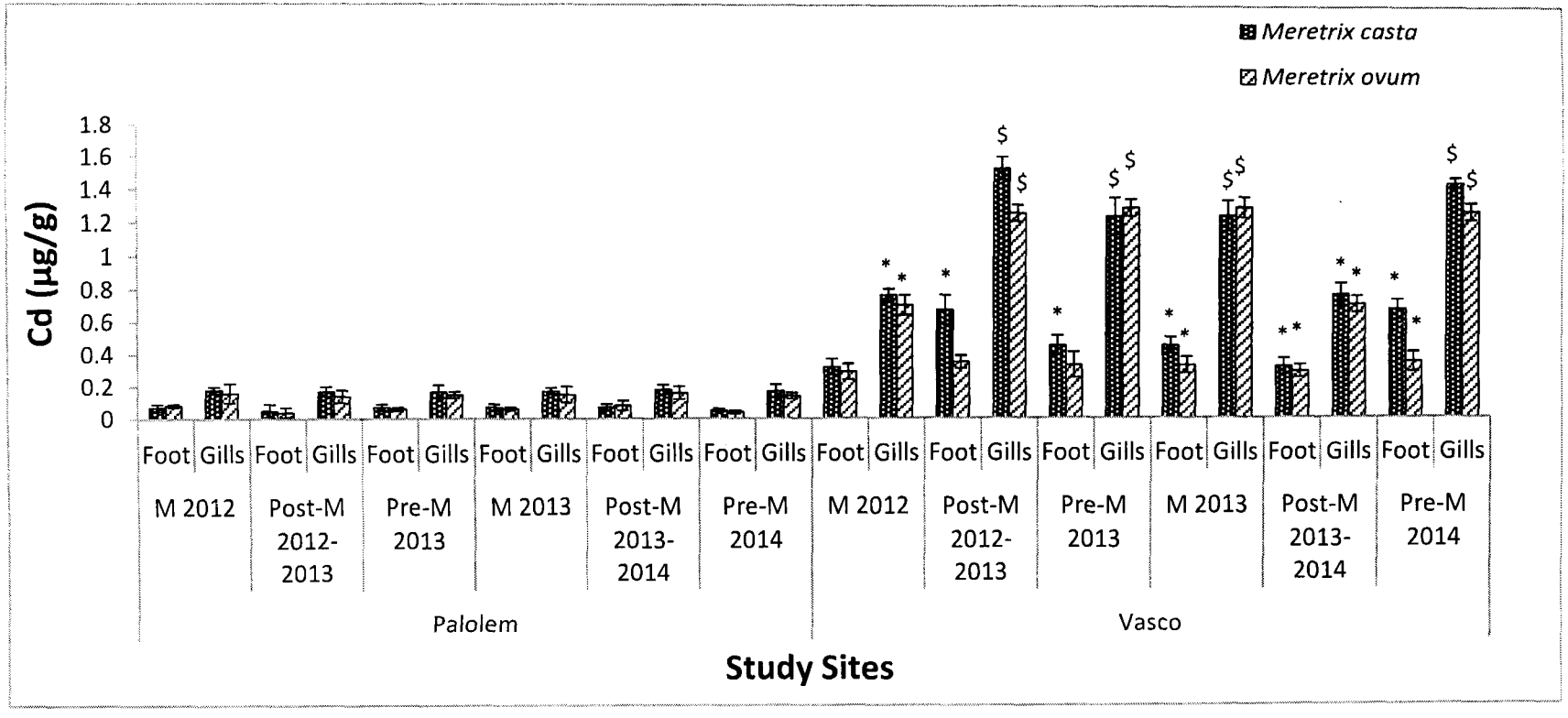
Note: M = monsoons, * $p < 0.05$

Fig. 2.29. Cu (Mean ± SD) in tissues of shellfish collected from various study sites in different seasons from June 2012 to May 2014



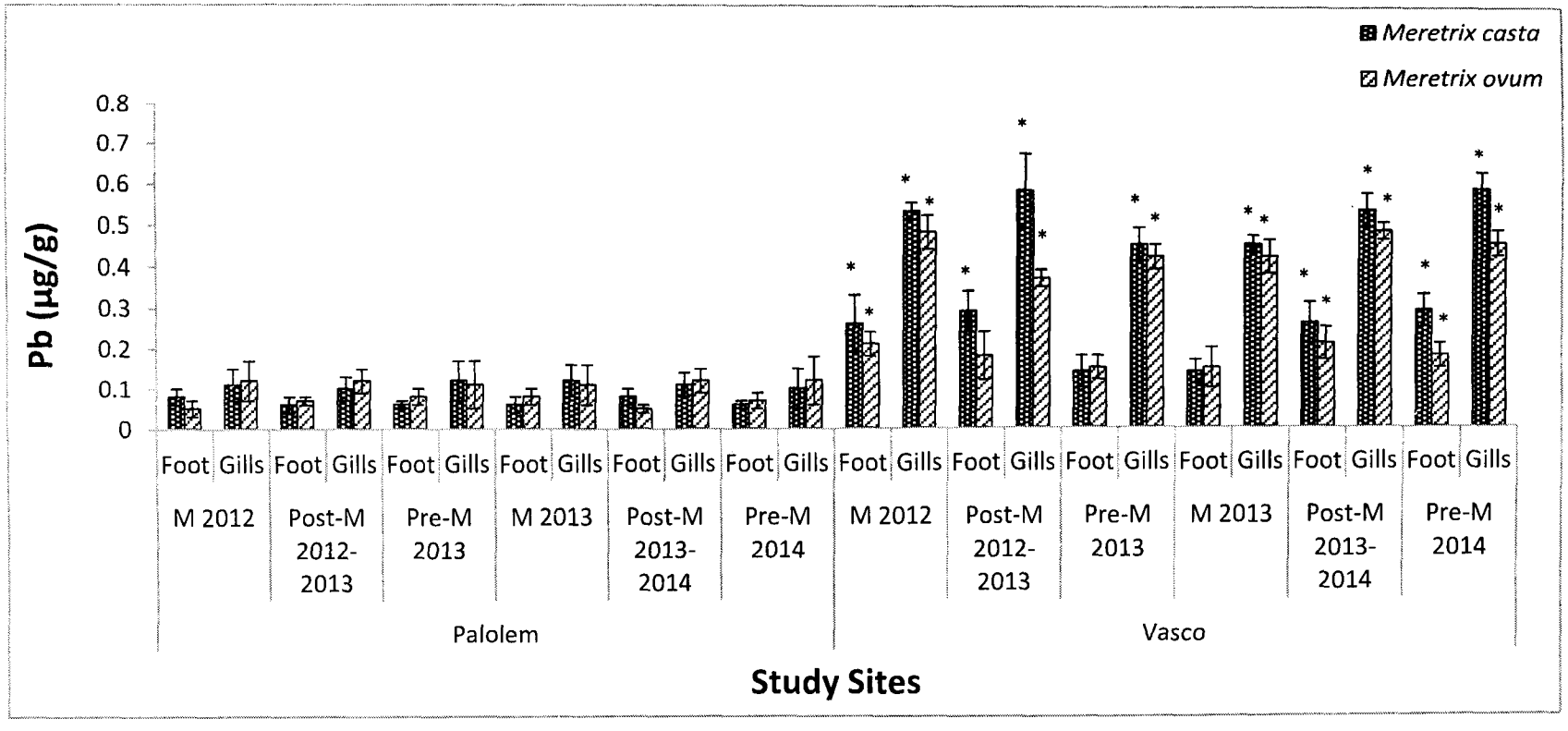
Note: M = monsoons, * $p < 0.05$

Fig. 2.30. Cd (Mean ± SD) in tissues of shellfish collected from various study sites in different seasons from June 2012 to May 2014



Note: M = monsoons, $ $p < 0.01$, * $p < 0.05$

Fig. 2.31. Pb (Mean ± SD) in tissues of shellfish collected from various study sites in different seasons from June 2012 to May 2014



Note: M = monsoons, * $p < 0.05$

Fig. 2.32. Principle Component Analysis (PCA) to reduce the number of variables that maybe inter-correlated

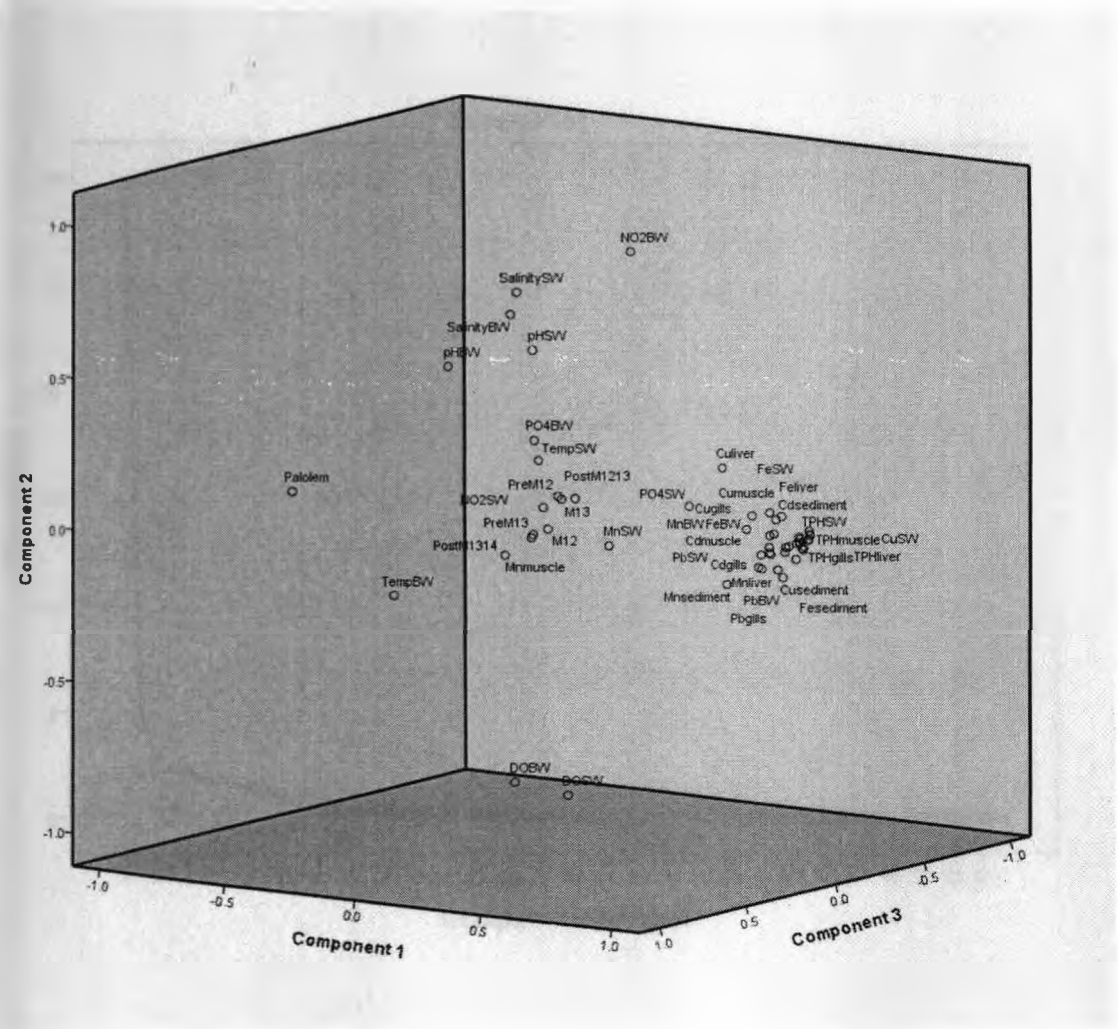


Fig. 2.33. Principle Component Analysis (PCA) depicting the components that are inter-correlated

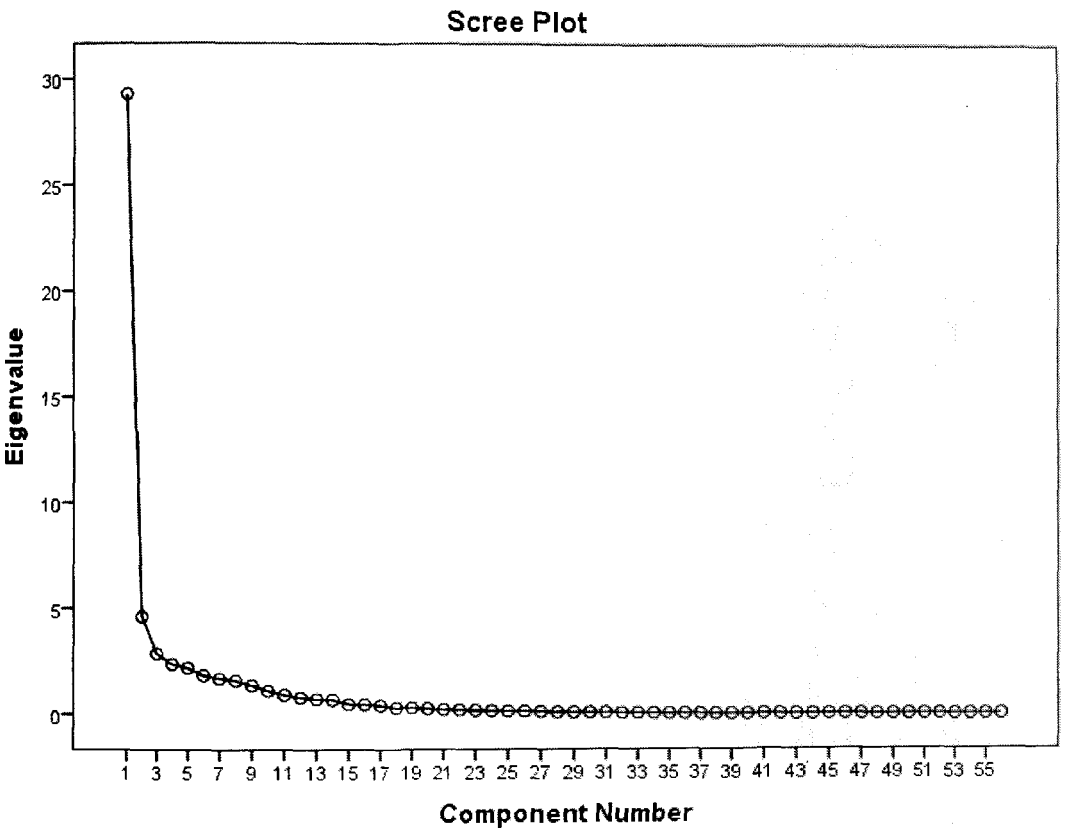


Table 2.1. Correlation coefficients (Pearson's) between DNA damage and levels of heavy metals in the tissues of finfish and shellfish

		TPH	Fe	Mn	Cu	Cd	Pb
Finfish	Muscle	0.88 #	0.84 #	-0.23	0.60 *	0.77 $	0.80 #
	Gill	0.87 #	0.87 #	0.83 #	0.43	0.74 $	0.76 $
	Liver	0.89 #	0.86 #	0.75 $	0.39	0.75 $	0.81 #
Shellfish	Foot	0.85 #	0.82 #	0.05	0.26	0.83 #	0.70 $
	Gill	0.90 #	0.86 #	0.19	0.68 *	0.88 #	0.63 *

Note: # $p < 0.001$, $ $p < 0.01$, * $p < 0.05$

Table 2.2. Multiple regression model for predicting the DNA damage using factor scores obtained from principal components analysis

Predictors	Coefficients	t-value	Significance (p)
Component 1	0.875 ± 0.015	16.91	<0.0001
Component 2	-0.016 ± 0.015	-0.30	0.763
Component 3	0.048 ± 0.015	0.92	0.359
Component 4	0.089 ± 0.015	-1.72	0.089
Component 5	-0.170 ± 0.015	-3.29	0.052
Component 6	-0.060 ± 0.015	-1.16	0.248
Component 7	-0.056 ± 0.015	-1.07	0.287
Component 8	0.143 ± 0.015	0.76	0.058
Component 9	0.032 ± 0.015	0.61	0.542
Component 10	0.049 ± 0.015	0.94	0.347

Table 2.3. Correlation matrix between the association of DNA damage and physico-chemical parameters

	MN	% tail DNA	Temp	pH	Salinity	DO	Phosphates	Nitrates
MN	-							
% tail DNA	0.85 #	-						
Temp	0.09	0.09	-					
pH	-0.08	-0.06	0.12	-				
Salinity	-0.45 *	-0.44 *	-0.09	0.04	-			
DO	-0.57 *	-0.59 *	-0.12	0.03	0.56 *	-		
Phosphates	-0.04	0.18	0.05	0.05	-0.12	0.11	-	
Nitrates	0.16	0.10	0.11	0.04	-0.06	0.07	-0.32	-

Table 2.4. Two-way analysis of variance (ANOVA) to assess the influence of sites and seasons on MN and % tail DNA

	F value			
	Site	Season	Site*Season	R square
MN	92.15 #	0.35	1.28	0.45
% tail DNA	113.22 #	0.32	1.12	0.55

Note: M = # $p < 0.001$, * $p < 0.05$

Plate 2.1. Finfishes selected for the present study

Common name: Sin Croaker **Local name**: *Dodyaro*

Classification

Kingdom	:	*Animalia*
Phylum	:	*Chordata*
Class	:	*Actinopterygii*
Order	:	*Perciformes*
Family	:	*Sciaenidae*
Genus	:	*Johnius*
Species	:	*J. dussumieri*

Common name: Threadfin sea catfish Local name: Sangott

Classification

Kingdom	:	*Animalia*
Phylum	:	*Chordata*
Class	:	*Actinopterygii*
Order	:	*Siluriformes*
Family	:	*Ariidae*
Genus	:	*Arius*
Species	:	*A. arius*

Plate 2.2. Shellfishes selected for the present study

Common name: Backwater / Estuarine Clam **Local name:** *Tisreo*

Classification

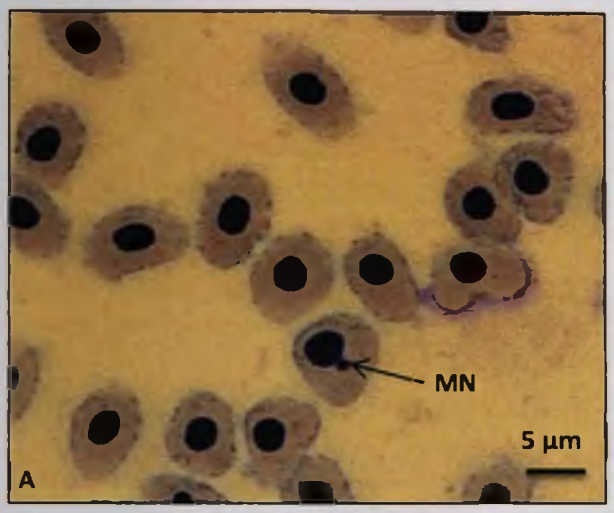
Kingdom : *Animalia*
Phylum : *Mollusca*
Class : *Bivalvia*
Order : *Venerida*
Family : *Veneridae*
Genus : *Meretrix*
Species : *M. casta*

Common name: Backwater / Estuarine Clam **Local name:** *Tisreo*

Classification

Kingdom : *Animalia*
Phylum : *Mollusca*
Class : *Bivalvia*
Order : *Venerida*
Family : *Veneridae*
Genus : *Meretrix*
Species : *M. ovum*

Plate 2.3. Micronuclei in the blood cells of **A.** *Johnius dussumieri* and **B.** *Arius arius* (Magnification 1000X)



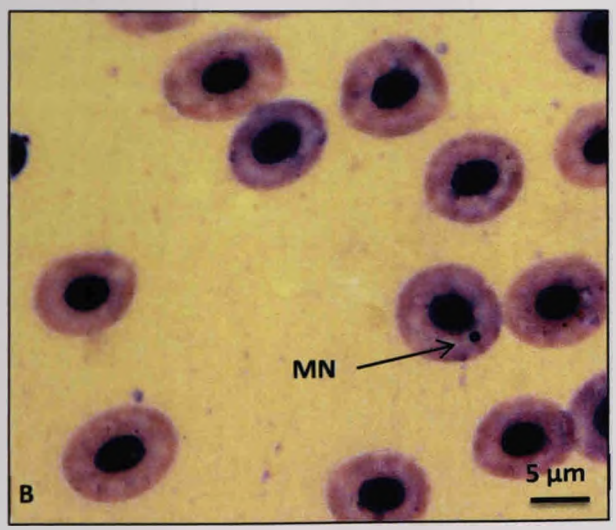
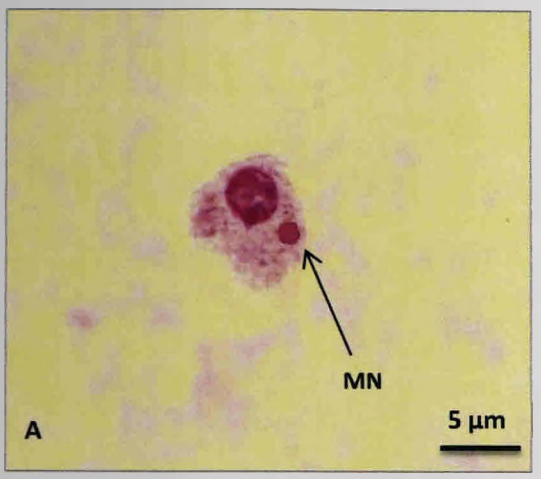


Plate 2.4. Micronuclei in the hemocytes of **A.** *Meretrix casta* and **B.** *Meretrix ovum* (Magnification 1000X)



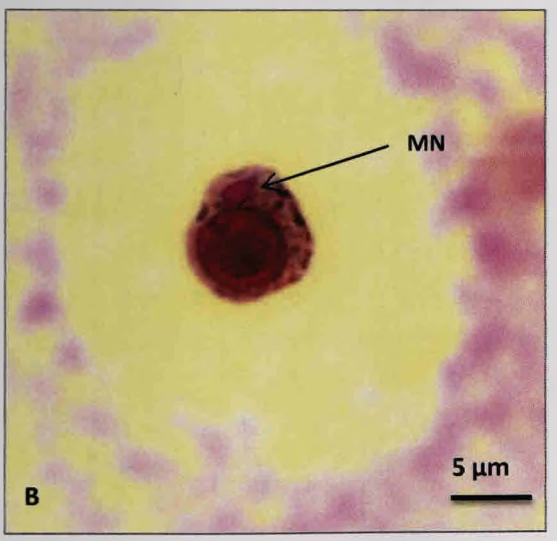
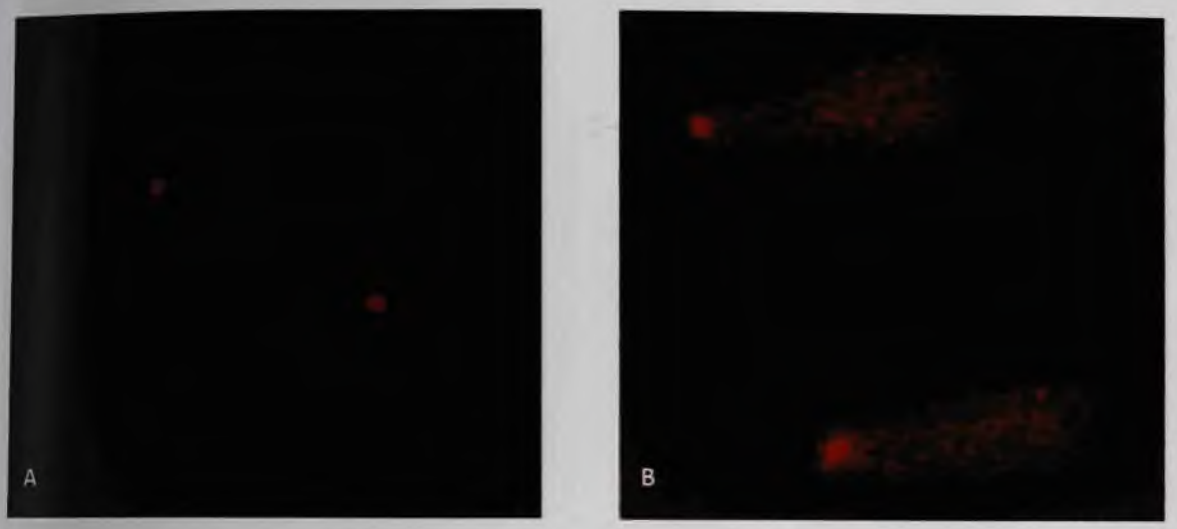


Plate 2.5. Comet assay **A.** Undamaged cells and **B.** Cells with DNA damage (Magnification 200X) **C.** Analysis of comets using CASP software



2.5. Discussion

The present study revealed that the finfishes collected from Vasco, Dona Paula and Miramar, as well as the shellfishes collected from Vasco contain high concentrations of TPHs and trace metals, which might have induced DNA damage, as evident by the MN test and comet assay. This can lead to the deterioration in the health of the fish and clam populations and consequently result in the decline in fish catches around this area. Further, this may also pose a threat to the health of fish consumers as well as the health and livelihood of the fishing communities at Vasco, Dona Paula and Miramar.

2.5.1. Micronucleus test

Several scientists have employed the micronucleus test to assess the genotoxicity of compounds released by industries into aquatic environments (Baršiene *et al.*, 2004; Cavaş and Ergene-Gozukara, 2005; Da Silva Souza and Fontanetti, 2006). Damaged chromosomes result from inefficient or incorrect DNA repair and/or from the presence of metals around the mitotic apparatus. This damage is expressed during cell division in the form of micronuclei and may represent an index of accumulated genotoxic agents (Kligerman, 1982). In the present study, the increased incidence of micronuclei in the blood cells of *A. arius* may possibly be due to the high concentrations of trace metals and TPHs at Vasco. Further, even an increased / long-term exposure to low concentrations of metals and TPHs in these water bodies might have contributed towards the induction of MN in the present study, as per the reports of Alink *et al.* (2007) wherein genotoxicants in water lead to an increase in the genotoxic effects in the cells of fish and other aquatic organisms.

Both the species of bivalves, *M. casta* and *M. ovum*, collected from Vasco showed a significantly high frequency of MN in the gill cells in all the seasons. This is in agreement

with several reports of genetic damage in the bivalves exposed to pollutants including trace metals, various hydrocarbons and biphenyls such as that of Schiedek *et al.* (2006), wherein they reported a significantly high frequency of micronuclei in blue mussels (*Mytilus edulis*) from a location polluted with metals, PAHs and organochlorines in the Baltic Sea. In the present study, a significant incidence of micronuclei was evident in the bivalves collected from petroleum polluted areas such as Vasco, Dona Paula and Miramar. These observations are in agreement with the studies of Baršiene *et al.* (2006b) in which they sampled blue mussels (*Mytilus* spp.) from different zones in the Baltic Sea and also reported a high frequency of micronuclei in the gill cells of mussels from areas polluted with petroleum products. Our results also find similarity to another study in which mussels (*Mytilus edulis*) collected from the Baltic Sea after an accidental oil spill showed significant elevation in the frequency of micronuclei compared to those collected before the oil spill (Baršiene *et al.*, 2012).

In the present study, the concentration of metals and petroleum hydrocarbons in the waters contribute to DNA damage such as MN in the regression model. Thus, the presence of these MN has been largely attributed to the presence of various xenobiotics in the environment. MN, which arise due to lagging DNA fragments or a whole chromosome, do not get incorporated during cell division and persist as a small body (micronucleus) in close proximity with the main nucleus and thus this assay is useful for any proliferating cell population (Bolognesi and Hayashi, 2011). The lagging DNA fragments / chromosomes arise as a consequence of long term exposure associated with an accumulated effect of genotoxic or clastogenic agents as also observed in the present study which either directly affect the

DNA or interfere with mitotic processes (Fenech *et al.*, 2003; Baršiene *et al.*, 2008; Bolognesi and Cirillo, 2014).

2.5.2. Comet assay

DNA damage as measured by the comet assay has been linked to a wide spectrum of genotoxic and cytotoxic compounds, such as PAHs and trace metals (Lee and Steinert, 2003). The amount of the DNA in the tail region (tail DNA) of a comet is commonly used for quantifying DNA strand breakage and represents the most reliable parameter (Mitchelmore and Chipman, 1998; Kumaravel and Jha, 2006). In this study, the fish collected from Vasco, Dona Paula and Miramar showed significant DNA damage in all seasons as compared to those collected from Palolem. These results are on par with a study by Sarkar *et al.* (2008) in which the DNA integrity in marine gastropods was found to be significantly damaged at Dona Paula and Vasco during the pre-monsoon, monsoon and post-monsoon periods. According to Theodorakis *et al.* (1994), chronic exposure to pollutants can lead to an accumulation of DNA strand breaks in aquatic organisms, such as fish, since their DNA-repair capacity is much lower compared to that of other species.

A significant increase in the % tail DNA was observed from the clams collected from Vasco (Fig. 3). This indicates the presence of a high level of DNA damaging agents, probably trace metals and various hydrocarbons which will lead to the loss of DNA integrity through DNA strand breaks at the individual cell level (Bolognesi and Cirillo, 2014). These observations are in agreement with the studies of Nigro *et al.* (2006) in which they observed a significant increase of DNA damage measured by the comet assay in *Mytilus galloprovincialis* from a polluted estuary in Italy. Our observation is also in agreement with Sarkar *et al.* (2008) who reported a decrease in DNA integrity in the gastropod, *Cronia contracta*, from Vasco and

were reported to be attributed to exposure to polycyclic aromatic hydrocarbons and metals such as Pb, Cu, Cd, Fe and Mn. Our results also find similarity with that of Sarkar *et al.* (2014) in which they reported a decrease in DNA integrity of another gastropod, *Morula granulata* from Hollant, which is located close to Vasco. The probable reason for the induction of DNA damage in aquatic organisms such as those in the present study is due to the formation of reactive oxygen species. Certain petroleum hydrocarbons constituents, such as benzo[a]pyrene (a polycyclic aromatic hydrocarbon), when absorbed by the organism can be converted to the reactive oxygen species (diol epoxide), and can form a stable adduct with DNA (Bihari and Fafandel, 2004). These ROS and DNA adducts can lead to changes in the conformation and functioning of the DNA further resulting in carcinogenesis (Monserrat *et al.*, 2007; Ziech *et al.*, 2010).

DNA damage by xenobiotics occurs in three basic steps, wherein the first is the formation of adducts with toxic molecules. The next stage, secondary modifications of DNA, includes single- and double-strand breakage, changes in DNA repair, base oxidation and cross-links. Xenobiotics may induce these secondary modifications by way of ROS production. In the third stage, cells show altered function, which can lead to cell proliferation and consequently, cancer (Monserrat *et al.*, 2007).

We also observed a significant positive correlation between the frequencies of MN and % tail DNA ($R = 0.85$, $p < 0.001$) which are represented in the form of scatter plots (Fig. 4). The MN test and the comet assay reflect different forms of environmental stress. The comet assay is able to detect repairable DNA damage such as DNA strand breakages, whereas the MN test detects more persistent DNA damage which are more difficult to repair (Hartmann *et al.*, 2001; Klobucar *et al.*, 2003). The positive correlation in our study may be due to the

conversion of the short term reversible damage to long term irreversible damage in the long run. The combination of bioaccumulated contaminants in the tissues of the bivalves could therefore lead to formation of complexes with metabolites, reactive oxygen species and subsequently DNA damage at a level higher than the effects of individual contaminants.

2.5.3. Trace metals and TPHs

Trace metals may settle on the surface sediments and can affect benthic feeders, such as catfish. Trace metals, especially Fe, Mn, Cd and Pb, which are found to contaminate the water and sediment at Vasco, could also be the cause of genotoxicity in *A. arius*. This is in agreement with the studies of Bellas *et al.* (2007) and Omar *et al.* (2012) which reported the genotoxic effects of trace metals, such as Cu, Zn, Fe, Mn and Pb, in marine and estuarine environments. The presence of high concentrations of these metals due to extensive mining activities and transportation of ores can be attributed to the stress caused in the fish. Our results also find similarity with that of Salem *et al.* (2014) in which roaches (*Rutilus rutilus*) collected from a landfill containing mining leachates such as Cd, Cu, Mn and Pb were found to exhibit significant DNA damage.

Metal-induced genotoxicity is predominantly due to inhibition of DNA repair process. Some metals such as chromium, nickel, arsenic, cadmium and cobalt have long been recognized as human and/or animal carcinogens (Hartwig and Schwerdtle, 2002). Trace metals which may be genotoxic when accumulated at a high concentration in the tissues of aquatic organisms can have a profound effect on the integrity of their DNA leading to single or double strand breaks (Bolognesi *et al.*, 2004). Trace metals can also stimulate the formation of reactive oxygen species (ROS) (Verlecar *et al.*, 2008) and can therefore indirectly cause damage to DNA, alter proteins and induce lipid peroxidation resulting in decreased nutritive value and

disease following consumption (Valko *et al.*, 2006; Qian *et al.*, 2008; Regoli and Giuliani, 2014). Another consequence of bioaccumulation of metals is the effect on the physiological well-being of the organism which in turn also affects its meat quality (Orban *et al.*, 2002; Pampanin *et al.*, 2005).

Our results on DNA damage in fish are also on par with those of de Andrade *et al.* (2004) in which they observed genotoxic damage measured as single-strand DNA breaks employing the comet assay in the tissues of mullet and sea catfish from polluted sites in Brazilian rivers. Further, they also reported the association between the concentrations of hydrocarbons, trace metals, pH and water temperature and the levels of DNA damage. The multiple regression analysis conducted in the present study revealed that high levels of DNA damage are due to the accumulation of the pollutants from the environment. Trace metal accumulation in different tissues at high concentrations could occur due to various reasons. Trace metal accumulation in the liver could occur during metal detoxification in fish; it may occur in the muscle tissues because of absorption of residues through the intestinal walls, and it occurs in the gills due to metal complex formation with mucus on the gill lamellae (Taylor *et al.*, 1985; du Preez *et al.*, 1993). Particulate trace metals (nano-sized) may interact with fish or other organisms in four ways: 1) adsorption to the surface i.e. cell, organ or whole body 2) cellular internalization 3) dissolution of ions from the NP and 4) mechanistic nano-effects, such as formation of reactive oxygen species (ROS) (Baker *et al.*, 2014). Trace metals like cadmium and copper may also affect embryonic or larval stages and may cause increased mortality, altered body size as well as morphological deformities (Witeska *et al.*, 2014). Our findings can also be compared with the results of Costa *et al.* (2011), in which the exposure to

GOVERNMENT OF GOA,
OFFICE OF THE EXECUTIVE ENGINEER,
WORKS DIVISION I(Bldgs) ,
PUBLIC WORKS DEPARTMENT,
PANAJI - GOA.

CERTIFICATE " C "

Name of Work:- Assessment of concrete quality for A, B, C type Quarters at Altinho, Bhatulem, Panaji-Goa.

It is hereby certified that there is no violation of CRZ guidelines. No sand dunes are affected and no mangroves cutting is involved in the above mentioned work.

ASSISTANT ENGINEER
SD -III, WD-I (Bldgs), PWD
Altinho - GOA

EXECUTIVE ENGINEER
W.D.I(Bldgs), PWD
PATTO, PANAJI -GOA

sediments contaminated by trace metals induced DNA fragmentation and clastogenesis in Senegalese soles.

The presence of TPHs in the tissues of the fish as well as the bivalves in the present study also indicate the presence of carcinogenic components in these hydrocarbons such as benzo[α]pyrene (B[α]P). B[α]P, a representative polycylic aromatic hydrocarbon (PAH) present in crude oil fractions, is reported to be converted at the cellular level to the ROS, diol-epoxide (BaPDE), which can form stable adduct with DNA, resulting in DNA strand breaks (Pisoni *et al.*, 2004; Bihari and Fafandel, 2004). Balk *et al.* (2011) reported the presence of PAH metabolites in the bile of fish collected from the North Sea which is known to be polluted with petroleum hydrocarbons. These metabolites, besides being genotoxic can exert other toxic effects in the rest of the body (Aas *et al.*, 2000) and may also affect the nutritive quality and palatability of fish / bivalve meat (Ansari *et al.*, 2012).

Seasonality plays an important role in the bioaccumulation of environmental contaminants and in turn may affect DNA damage (Catsiki and Florou, 2006, Hallanger *et al.*, 2011, Sacchi *et al.*, 2013). Conversely, based on the two-way ANOVA in our present study, seasonality does not seem to affect DNA damage (F=0.35; 0.32). Our results are in agreement with that of Singh and Hartl (2012) wherein they reported that changes in environmental conditions do not affect DNA damage. This could be due to species-specific mechanisms as both clams and mussels are well adapted to the intertidal environment (Singh and Hartl, 2012; Martins and Costa, 2015).

In the present study, the presence of pollutants especially TPH, Fe, Mn, Cd and Pb in the tissues could be associated with genotoxicity in these organisms. The possible reason for this association could be attributed to the intensity of mining and harbor activities occurring at

Vasco. In addition, although a significant association is seen between the pollutants and genotoxicity, it may also be noted that the presence of other contaminants in lesser concentrations may also influence the genotoxicity in these fish and can be determined by further studies using both field and laboratory based experiments.

2.6. Conclusion

Although the concentrations of contaminants in these fish are much below the permissible limits recommended by EC (2005), FAO (1983) and IAEA (2003), it may be noted that these fish are consumed throughout the year by the Goan population and may lead to subsequent bioaccumulation and genotoxicity in humans as well. The data for genotoxicity recorded in these fish can be used as reference values for future genotoxicity, biochemical or physiological studies at these sites. Laboratory based studies can be conducted to confirm the genotoxicity of these pollutants either singly or in association with each other and the exact pathways of genotoxic damage can be deduced as future work. As the industrial wastewaters in Goa are not properly processed and treated before release into the environment, a treatment method should be devised and enforced by the Government and Coast Guard. Pollution indicators and genotoxicity tests, combined with other physiological or biochemical parameters represent an essential tool for efficient monitoring of aquatic ecosystems in Goa.

Chapter 3:

Laboratory confirmation of genotoxic potential of the pollutants

3.1. Introduction

Molluscs are popular bioindicators due to their ubiquitous distribution in both terrestrial and aquatic environments around the world. They are associated with various ecological roles and are particularly important in the functioning of ecosystems such as litter decomposition and contribution to huge amounts of the biomass at the different trophic levels in ecosystems (from primary consumers to top predators). Many groups of higher animals including echinoderms, fish, birds and mammals feed on molluscs. Of the seven molluscan classes, gastropods and bivalves are the major classes of molluscs followed by polyplacophorans, cephalopods, scaphopods, aplacophorans and monoplacophorans. A majority of the gastropods and bivalves exhibit extremely limited mobility or are completely sessile as adults. Therefore, because of their sedentary habit, these molluscs can precisely represent the contamination of their habitat ideally.

Most gastropod and bivalve species used for biomonitoring and bioindication purposes are relatively large and therefore easy to handle. Consequently, they can be used both under laboratory and field conditions, for active and passive biomonitoring. Due to the lack of an exoskeleton, molluscs are in direct contact with the ambient medium (water or soil). Therefore, chemicals can be taken up not only from the diet (via the gastro-intestinal tract) but also additionally from ambient water or soil via the integument, including the respiratory organs in aquatic species, resulting in greater accumulation potency for contaminants.

Compared with other invertebrate groups like arthropods and vertebrates, molluscs exhibit a limited ability to excrete pollutants via their kidneys or other excretory organs and tissues, to metabolize organic chemicals and physiologically to inactivate toxic heavy metals, e.g. by

the formation of and binding to metallothioneins (Lee, 1985; Berger *et al.*, 1995; Legierse *et al.*, 1998). As a consequence, molluscs attain higher bioaccumulation or bioconcentration factors for many toxicants than other systematic groups. Therefore, pollutants might exhibit negative impacts on molluscs even at lower environmental concentrations than on other invertebrates or vertebrates, facilitating their use as a kind of ecological early warning system. Consequently, biological effects of environmental stress in general and of contaminant exposure in particular are measurable at various levels of biological organisation (from molecules to communities). Most molluscs are model organisms for ecotoxicological research, especially as test animals and for environmental monitoring.

Generally, metal accumulation by such organisms is favoured by their limited ability to excrete these contaminants directly after their uptake and also by efficient physiological inactivation mechanisms, such as intracellular compartmentalization, or metal inactivation by binding to metallothioneins. Such biological accumulators have often been used as accumulation indicators of environmental metal pollution. Ideally, metal concentrations in the animal's body reflect environmental pollution levels quantitatively. According to the US EPA, shellfish can take up metals by several mechanisms: (1) ingestion of particulate substances from suspended material (2) ingestion of food material that have acquired these metals (3) uptake by exchange onto mucous sheets (4) incorporation of these metal ions into physiologically important systems and (5) formation of metal complexes with other organic molecules (EPA, 1971).

Since 1976, bivalves have been used to assess the levels of contamination in marine ecosystems, and certain systematic groups, notably mussels and oysters, have been extensively studied worldwide (Phillips and Rainbow, 1994). Their main advantage is that

the concentration of contaminants in the tissues of these organisms provide a time-integrated measure of pollutant bioavailability, responding essentially to that fraction of the total environmental load, which is of direct ecotoxicological relevance.

3.2. Review of Literature

The use of molluscs, particularly bivalves as "sentinels" is a popular approach for detecting various kinds of pollution of the environment (Zuykov *et al.*, 2013). Bivalves are widely used in biomonitoring programs due to their association with the estuarine sediment, filter-feeding habit and ability to bioaccumulate various pollutants.

A number of reports are available on the genotoxic effect of different heavy metals in various animal models including fishes and molluscs. In fish, acute and sub-chronic exposure to Cd leads to alterations of gill epithelium, liver and kidneys (Thophon *et al.*, 2003; Jebali *et al.*, 2006). Exposure to Cd induced reduction of growth rate and mortality of molluscs due to impairment of several metabolic functions (Ivanina *et al.*, 2010; Nicosia *et al.*, 2015). Several studies have also reported the genotoxicity of Cd in various animal models (Zharkov and Rosenquist, 2002, Cavaş *et al.*, 2005, Fourie *et al.*, 2007; Çelik *et al.*, 2009; Pavlaki *et al.*, 2016). The effect of Cd on DNA may be indirect, via the action of reactive oxygen species and thus leading to oxidative DNA damage (Liu *et al.*, 2009).

Lead (Pb), another heavy metal is also known to induce DNA damage in aquatic organisms (Ferraro *et al.*, 2004; Monteiro *et al.*, 2011; Ambreen and Javed, 2016; Di Donato *et al.*, 2016). In bivalves, Pb induces DNA damage (Black *et al.*, 1996; Sohail *et al.*, 2017), causes oxidative stress (Zhang *et al.*, 2010), affects gill structure (Domouhtsidou and Dimitriadis, 2000) and affects embryogenesis (Wang *et al.*, 2009).

The effects of copper on aquatic organisms include DNA damage (Gabbianelli *et al.*, 2003) and oxidative stress (Al-Subiai *et al.*, 2011). The accumulation of copper may also cause alterations in the gills and digestive gland structure of bivalves (Jing *et al.*, 2006; Sabatini *et al.*, 2011) and may also hinder immune activity (Nicholson, 2003; Parry and Pipe, 2004). Besides these effects, burrowing behaviour may also be induced in bivalves as a stress response to increasing concentrations of Cu (Bonnard *et al*, 2009).

Iron dissolved in sea water was found to induce oxidative damage in the bivalve *Mya arenaria* which could further lead to DNA damage in the soft tissues (González *et al.*, 2010). Taze et al. (2016) also reported oxidative damage in the hemocytes of mussels (*Mytilus galloprovincialis*) exposed to iron oxide nanoparticles for 1, 3 and 7 days. Increased production of reactive oxygen species, lipid peroxidation and DNA damage was observed as a consequence of this oxidative damage.

Oysters (*Crassostrea virginica*) exposed to 1mM of manganese for three days were reported to exhibit neurotoxicity due to the impairment of the dopaminergic innervation in the cilia of the gills (Martin *et al.*, 2008; Nelson *et al.*, 2010). King *et al.* (2008) also observed a decrease in the levels of dopamine in the cerebral ganglia, visceral ganglia and gills of *Crassostrea virginica.* Free ions of manganese were also found to alter valve movement behaviour in the bivalve *Velesunio angasi* (Markich *et al.*, 2000)

Varotto *et al.* (2013) reported a significant increase of DNA damage in the form of micronuclei in the gills of *Mytilus galloprovincialis* exposed to nanomolar concentrations of Cd, Cu and Hg. Significant DNA damage in the form of % tail DNA was also observed in in the hemolymph, gill cells and digestive gland cells of Manila clams (*Tapes semidecussatus*)

exposed to estuarine sediments contaminated with toxic compounds such as heavy metals (Coughlan *et al.*, 2002). Similarly, Al-Subiai *et al.* (2011) reported accumulation as well as significant DNA damage in the tissues of *Mytilus edulis* exposed to various concentrations of Cu. Exposure of *Mytilus galloprovincialis* to various concentrations of copper oxide nanoparticles for a period of 15 days induced genotoxic effect in their hemocytes (Gomes *et al.*, 2013). Clams (*Corbicula fluminea*) exposed to various concentrations of aqueous cadmium (3, 6, 12 and 25 ppb) exhibited significant DNA strand breaks and impairment of DNA integrity (Barfield *et al.*, 2001). Regoli *et al.* (2004) reported a significant decrease in DNA damage as measured by the comet assay in *Mytilus galloprovincialis* from Genova harbor in Italy which is highly contaminated with metals such as Cd, Fe, Mn, Pb and Cu. Hamoutene *et al.* (2002) exposed the mussel *Mytilus edulis* and the clam *Mya arenaria* to water soluble fractions of crude oil and observed single-strand DNA damage in the form of % tail DNA in the digestive gland cells. Pérez-Cadahía *et al.* (2004) exposed the mussel *Mytilus galloprovincialis* to oil from the Prestige oil spill and reported a significant increase in DNA damage. This DNA damage was also found to be highly correlated with the tissue concentrations of accumulated total petroleum hydrocarbons after exposure. In another study, Martins *et al.* (2013) reported single-strand DNA damage in the form of % tail DNA in *Ruditapes decussatus* exposed to sediments spiked with environmentally-relevant concentrations of polycyclic aromatic hydrocarbons. They also reported that low doses of both carcinogenic as well as non-carcinogenic PAHs were able to induce genotoxicity in these clams. Exposure to environmentally relevant concentrations of Cd, benzo[a]pyrene and combination of both these pollutants were found to significantly increase single-strand DNA damage in the form of % tail DNA in the gill cells and hemocytes of the zebra mussel

(*Dreissena polymorpha*) (Vincent-Hubert *et al.* 2011). Baršiene *et al.* (2006c) reported the induction of MN in the gill cells of the duck mussel (*Anodonta anatina*) that were exposed to different concentrations of crude oil from an oil-well in Lithuania. DNA damage as % tail DNA and MN was also induced in the Asian clam *Corbicula fluminea* exposed to the water-soluble fraction of gasoline (Fedato *et al.*, 2010). In another study, mussels (*Mytilus galloprovincialis*) exposed to various concentrations of benzo[a]pyrene, one of the carcinogenic hydrocarbons in petroleum products, exhibited significant single-strand DNA damage as % tail DNA and % MN which increased over time (Banni *et al.*, 2010). DNA damage was also reported in the gonads of the scallop *Chlamys farreri* exposed continuously for 15 days to various concentrations of benzo[a]pyrene (Jing-jing *et al.*, 2009). They also observed alterations and degeneration of the oocytes of the ovaries indicating the toxic effects of B[a]P on germ cells.

3.3. Materials and Methods

3.3.1. Quality assurance and quality control

The appropriate quality assurance methods of sample preparation, handling and preservation were carried out in accordance with US EPA procedures. All chemicals used were of analytical grade from Himedia (Himedia, India) unless specified otherwise.

3.3.2. Maintenance of *Meretrix casta*

Meretrix casta (Estuarine backwater clam) was selected for the present study as it is consumed by a majority of the Goan coastal population and also due to its availability in the Goan estuaries throughout the year. The bivalves (both sexes) were collected during the monsoon season in 2014 from the intertidal zone with the help of skilled local fishermen

from Palolem in Goa. This site is a clean, pristine beach with no known industrial activity or anthropogenic stress (Sarker and Sarkar 2015). They were stored in a bucket with water from the study site and transported alive to the laboratory. They were then allowed to acclimatize in ordinary seawater from Palolem for 30 days. The water conditions were maintained as follows: temperature 25°C, pH 7.5 and salinity 25 ppt. The water was changed once daily to reduce fecal contamination.

3.3.3. Treatment schedule

Bivalves were distributed in groups, each containing 10 individuals and were used for dose-response studies. Concentrations of total petroleum hydrocarbons [crude oil], iron [Fe_2O_3], manganese [MnO_2], copper [$CuCl_2$], cadmium [$CdCl_2$] and lead [$Pb(CH_3COO)_2$] were selected based on the environmental levels reported by us in the field studies. For each pollutant, five different concentrations were chosen denoting a range of doses. The range of concentrations for total petroleum hydrocarbons was 5, 10, 15, 20 and 25 µg/L. The range for iron was 2, 4, 6, 8 and 10 µg/L whereas that of manganese, copper, cadmium and lead was 0.5, 1, 1.5, 2 and 2.5 µg/L. A group of bivalves were exposed to a combination of all these pollutants to simulate the nature of pollution which prevails in the Goan coast. The concentrations of these pollutants in the mixture representing low, medium and high doses are given in table 3.1. The bivalve groups were exposed to the pollutants for a period of 15 days. The entire water was discarded and renewed daily along with the dose of the respective pollutant. A group of bivalves were maintained in parallel without any pollutant exposure and served as the negative control.

Table 3.1. Concentration of pollutants in the mixture exposed to *M. casta*

Dose	Concentration of pollutants (µg/L)					
	TPH	Fe	Mn	Cu	Cd	Pb
Low	5	2	0.5	0.5	0.5	0.5
Medium	15	6	1.5	1.5	1.5	1.5
High	25	10	2.5	2.5	2.5	2.5

3.3.4. Cell viability

Prior to the comet assay and micronucleus test, the cell count and cell viability of the hemolymph and gill cells were checked to ensure that there were enough living cells to perform the assay employing the trypan blue dye exclusion test. The samples showing more than 90% viability and a cell count of a minimum of 10^6 cells/ml were used for the tests.

3.3.5. Micronucleus Test

Hemolymph was withdrawn with the help of a syringe from the posterior adductor muscle and was smeared immediately on clean slides. Then the bivalves were opened and the gills were dissected out. The micronucleus (MN) test was performed following the protocol outlined by Baršiene et al. (2008). A portion of the dissected gill tissue was placed in a drop of acetic acid methanol mixture (1:3) on a clean glass slide and was then gently nipped with tweezers for a few minutes. The resulting cell suspension was then gently smeared and air-

dried. The smears were fixed in methanol for 10 min, stained with 5% Giemsa for 15 mins and allowed to dry. The frequency of micronuclei (MN) was recorded by scoring 2,000 intact cells per bivalve at 1000x magnification using an Olympus BX53 trinocular research microscope. Micronuclei (MNi) were identified according to the following criteria: (1) spherical or ovoid-shaped extra nuclear bodies in the cytoplasm (2) a diameter of 1/3 - 1/20 of the main nucleus (3) non-refractory bodies (4) colour texture and optical features resembling those of the nucleus, and (5) the bodies completely separated from the main nucleus.

3.3.6. Single Cell Gel Electrophoresis (Comet Assay)

The comet assay was carried out according to the protocol of Lee and Steinert (2003). All steps were carried out in dim light to prevent the photo-oxidation of DNA. Gill tissue (0.1g) was gently homogenized with phosphate buffer saline (pH 7.4) and the resulting cell suspension was filtered through a muslin cloth. This cell suspension was then embedded in low melting agarose (LMA) on frosted microscopic slides. The cells were then lysed by placing the slides in a cold lysing solution (2.5M NaCl, 100mM Na_2EDTA, 10mM Tris, 10% DMSO and 1% Triton-X pH 10) at 4°C, overnight. Following lysis the slides were placed in unwinding buffer (electrophoresis buffer, pH 10) for 15-20 min to allow the DNA to unwind. Electrophoresis was then performed for 30 min at 300 mA, 25 V (Biorad electrophoresis unit). The slides were placed in neutralization buffer (400mM Tris base, pH 7.5) for 5 min. The gel containing DNA was stained with ethidium bromide and examined using a fluorescence microscope (Olympus BX53) with a green filter at 200x magnification. Randomly selected non-overlapping cells were screened and their comets were analyzed with the help of computer software, CASP (Konca et al. 2003) and the % tail DNA was recorded.

The % tail DNA is the amount of DNA (in percent) present in the tail of the "comet" and is used as a measure of DNA damage (Kumaravel and Jha 2006).

3.4. Results

The dose response data of the MN test in bivalves exposed to various concentrations of TPHs are presented in figure 3.1. The incidence of MN was found to increase in a concentration-dependent manner in the hemocytes of bivalves exposed to various concentrations of TPH (fig. 3.1.A). However, significant increase ($p < 0.05$) was observed only at 15, 20 and 25 µg/L concentrations of TPH. In the gill cells, significant MN ($p < 0.05$) were observed at the 20 and 25 µg/L concentrations. The % tail DNA was also found to increase in the TPH-exposed bivalves in a concentration-dependent manner and is represented in fig. 3.2. Significant increase of the % tail DNA was observed in the hemocytes as well as the gill cells of the bivalves exposed to 10, 15, 20 and 25 µg/L of TPHs ($p < 0.05$, $p < 0.01$).

The data of the MN test in bivalves exposed to various concentrations of iron oxide are represented in fig 3.3. Bivalves exposed to various concentrations of iron oxide did not show significant induction of MN in the hemocytes (fig. 3.3.A). Similarly, significant MN was not induced in the gill cells except at the highest concentration (10 µg/L, $p < 0.05$) of iron oxide (fig. 3.3.B). The % tail DNA in hemocytes as well as gill cells of the bivalves exposed to various concentrations of iron oxide are represented in fig. 3.4. Significant % tail DNA ($p < 0.05$) was observed only at the 10 µg/L concentration of iron oxide in both the hemocytes and the gill cells.

The induction of MN in the cells of *M. casta* exposed to various concentrations of manganese dioxide is represented in fig. 3.5. The hemocytes of *M. casta* showed significant MN ($p < 0.05$) only at the highest concentration of manganese dioxide (2.5 µg/L) (fig. 3.5.A). In case of the gill cells, statistical significance ($p < 0.05$) was observed at 2 and 2.5 µg/L concentrations of manganese dioxide (fig. 3.5.B). The data of % tail DNA observed in the cells of *M. casta* exposed to manganese dioxide are represented in fig. 3.6. The % tail DNA was significant in the hemocytes as well as the gill cells of the bivalves exposed to 2 and 2.5 µg/L of manganese dioxide ($p < 0.05$).

The data of the MN test in the cells of *M. casta* exposed to various concentrations of copper are represented in figure 3.7. In this group, none of the concentrations of copper were able to induce significant MN in the hemocytes or gill cells of bivalves. The data of % tail DNA in hemocytes as well as in the gill cells of *M. casta* exposed to copper are represented in fig. 3.8. DNA damage was also found to be insignificant compared to the control in both the hemocytes and gill cells.

The DNA damage as MN in the bivalves exposed to various concentrations of cadmium chloride are given in fig. 3.9. Significant MN ($p < 0.05$) was observed in the hemocytes at the 2 and 2.5 µg/L concentration of cadmium chloride. In the gill cells, significant MN ($p < 0.05$) was observed at the concentrations of 1.5, 2 and 2.5 µg/L (fig. 3.9.b). DNA damage as % tail DNA in the hemocytes of the bivalves exposed to cadmium chloride are represented in fig. 3.10. The increase in the % tail DNA in the hemocytes was significant ($p < 0.05$) at 1.5 and 2 µg/L and highly significant at 2.5 µg/L concentrations of Cd ($p < 0.01$). The % tail DNA in the gill cells also showed a similar trend at these concentrations of cadmium chloride (fig. 3.10.B).

The presence of MN in the hemocytes and gill cells of *M. casta* exposed to various concentrations of lead is given in fig. 3.11.A and B respectively. Statistically significant MN ($p < 0.05$) in both hemocytes and gill cells were induced only at the highest concentration of Pb (2.5 µg/L). The % tail DNA observed in the cells of *M. casta* exposed to various concentrations of Pb are represented in fig. 3.12. Significant DNA damage ($p < 0.05$) was observed in the hemocytes at the 2 and 2.5 µg/L concentration of Pb (fig. 3.12.A). However, in the gill cells (fig. 3.12.B), significance was observed at 1.5, 2 and 2.5 µg/L concentrations ($p < 0.05, p < 0.01$).

The data of the experiments involving the exposure of the bivalves to different combinations of the pollutants are given in fig. 3.13. and 3.14. In the hemocytes, significant MN ($p < 0.05$) was induced at the medium and high concentrations of the pollutant mixture, whereas in the gill cells, all the concentrations of the mixture were able to induce significant MN ($p < 0.05$). Similarly, in case of the comet assay, MN was found to be significant in the hemocytes at the low and medium concentrations ($p < 0.05$) and highly significant at the high concentration of the pollutant mixture ($p < 0.01$). Further, significance was also observed in the gill cells at the low concentration ($p < 0.05$) as well as the medium and high concentrations ($p < 0.01$) of the pollutant mixture.

Plate 3.1. *Meretrix casta* actively feeding in estuarine water under laboratory conditions

Plate 3.2. MN in the gill cell of *Meretrix casta* (Magnification 1000X)

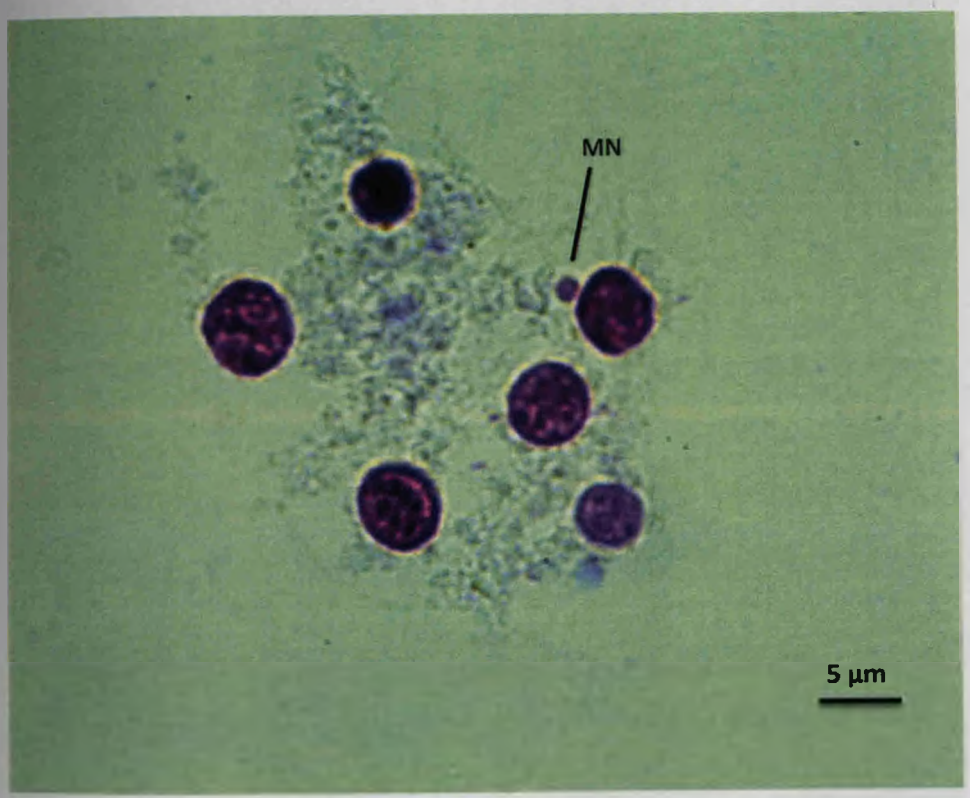
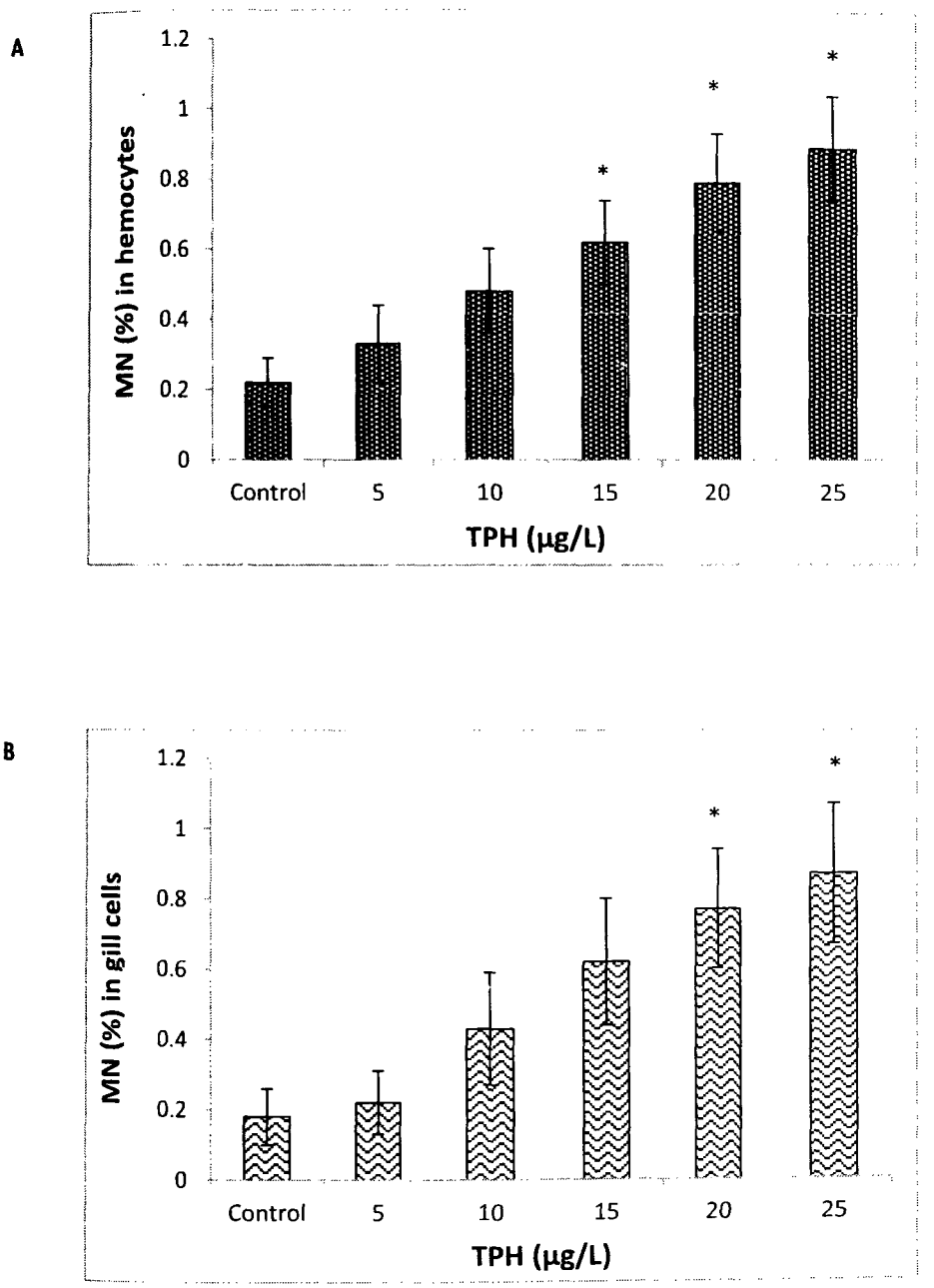
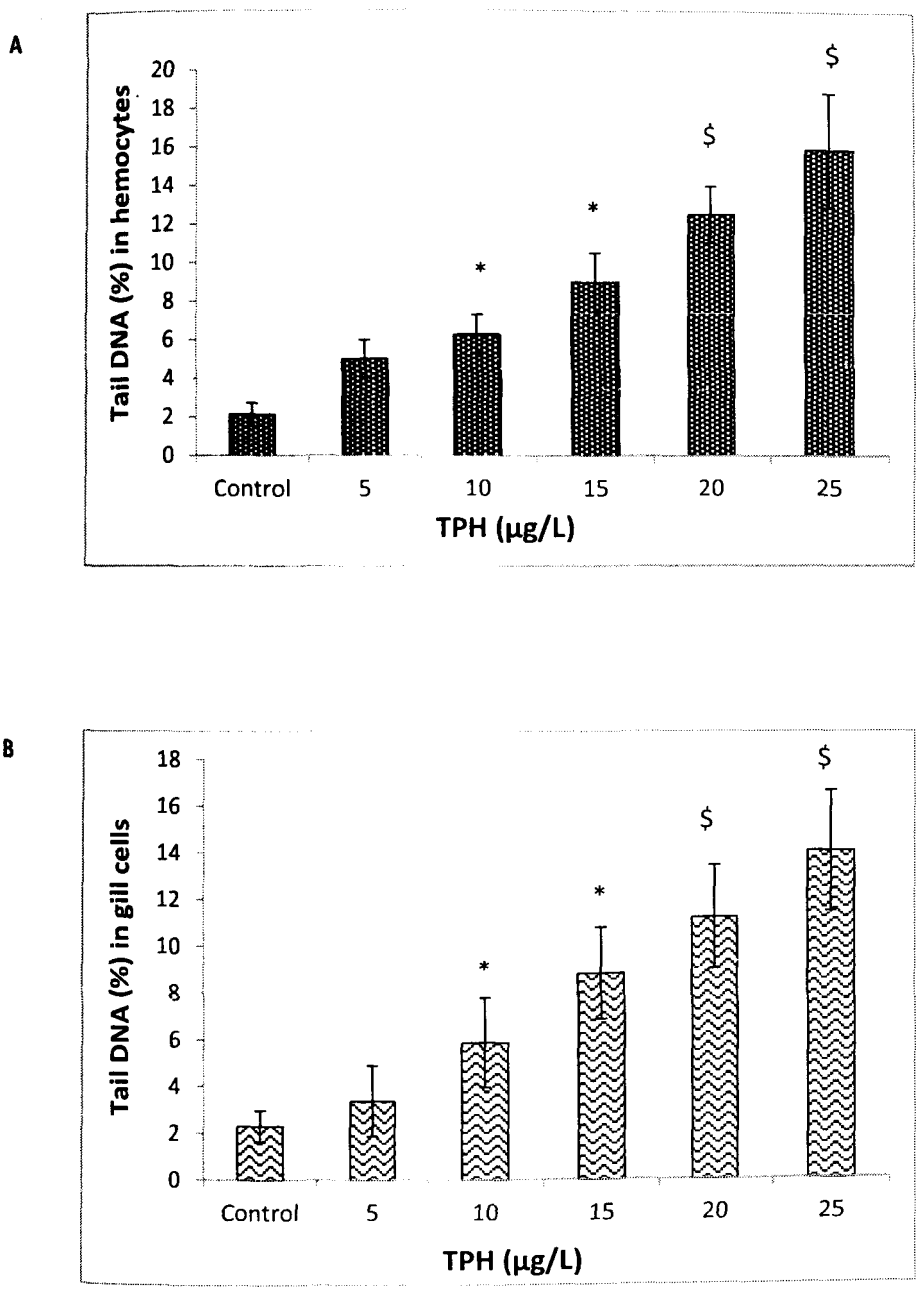


Fig. 3.1. MN (%) in the **(A)** hemocytes and **(B)** gill cells of *M. casta* (Mean ± SD) exposed to various concentrations of total petroleum hydrocarbons



Note: * $p < 0.05$

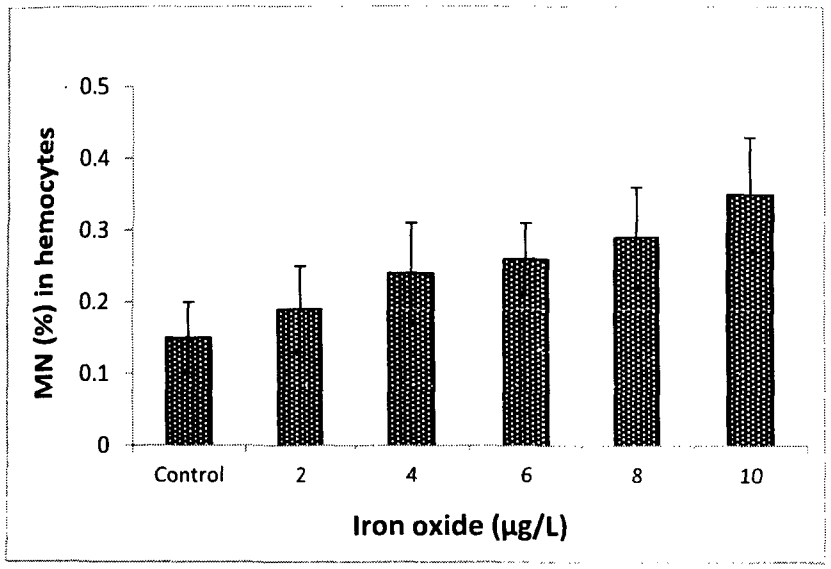
Fig. 3.2. Tail DNA (%) in the **(A)** hemocytes and **(B)** gill cells of *M. casta* (Mean ± SD) exposed to various concentrations of total petroleum hydrocarbons



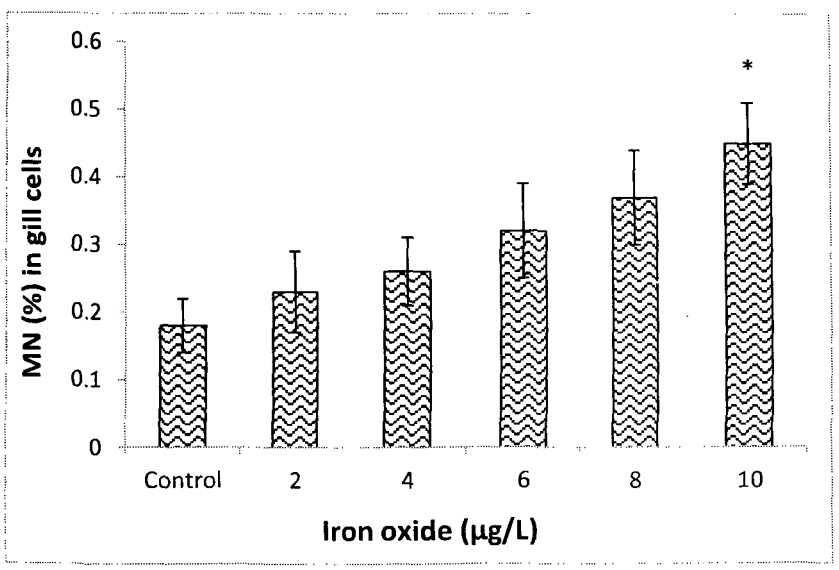
Note: * $p < 0.05$, $ $p < 0.01$

Fig. 3.3. MN (%) in the **(A)** hemocytes and **(B)** gill cells of *M. casta* (Mean ± SD) exposed to various concentrations of iron oxide

A



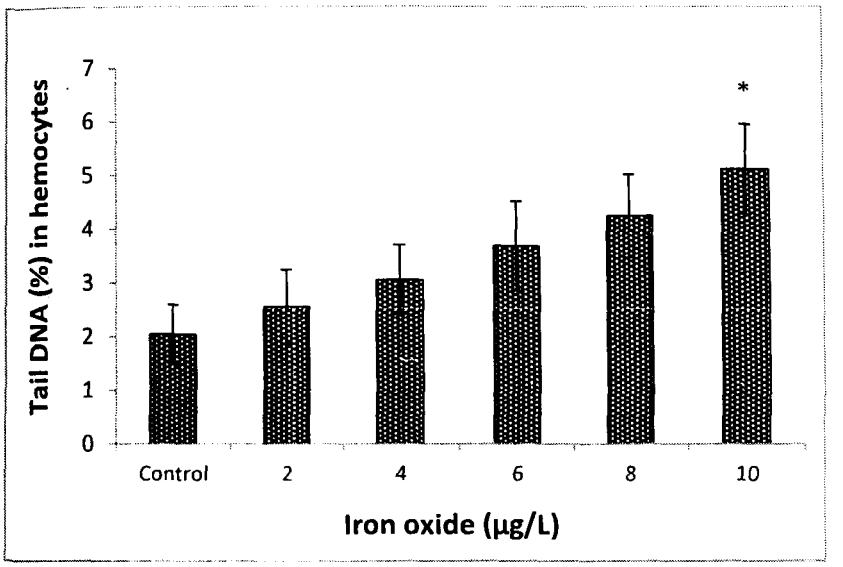
B



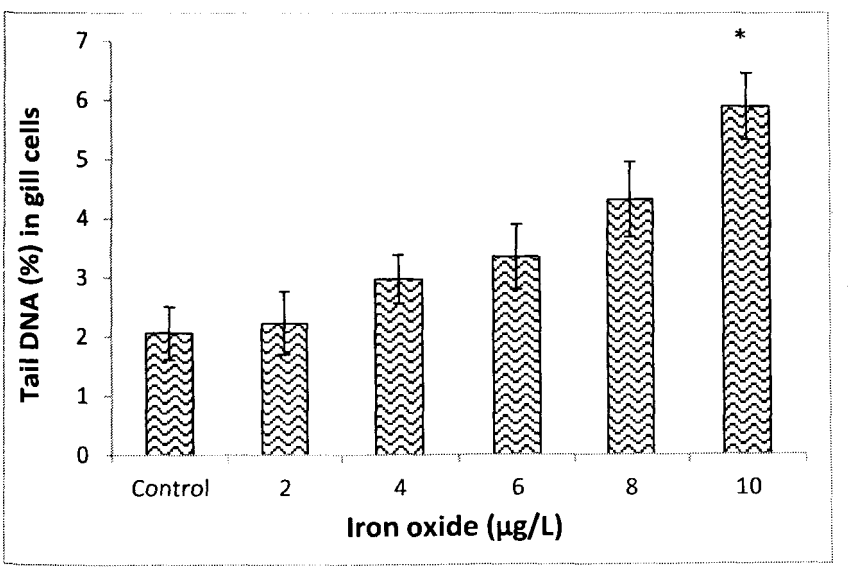
Note: * $p < 0.05$

Fig. 3.4. Tail DNA (%) in the **(A)** hemocytes and **(B)** gill cells of *M. casta* (Mean ± SD) exposed to various concentrations of total petroleum hydrocarbons

A



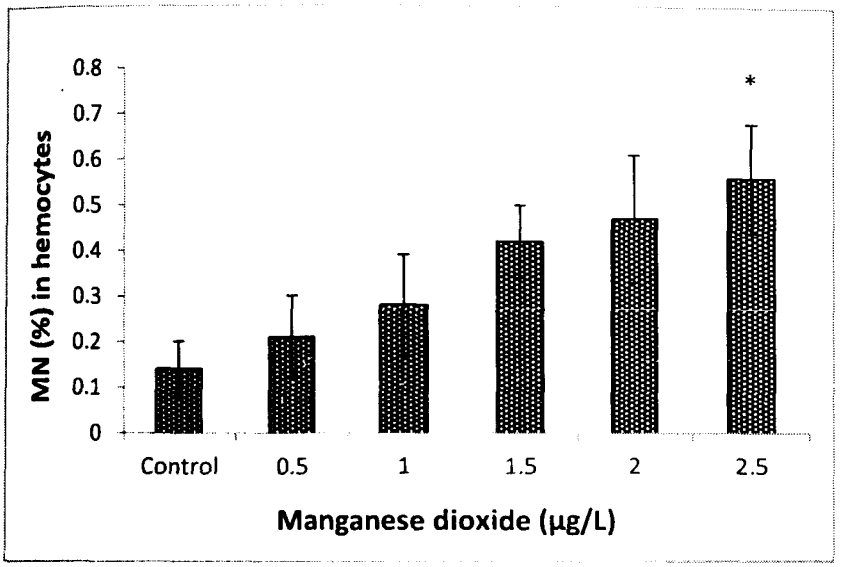
B



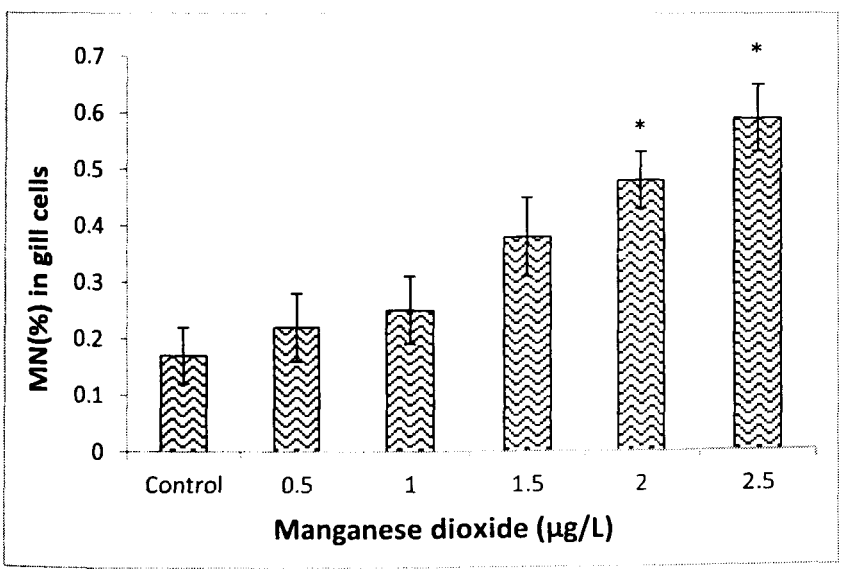
Note: * $p < 0.05$

Fig. 3.5. MN (%) in the (A) hemocytes and (B) gill cells of *M. casta* (Mean ± SD) exposed to various concentrations of manganese dioxide

A



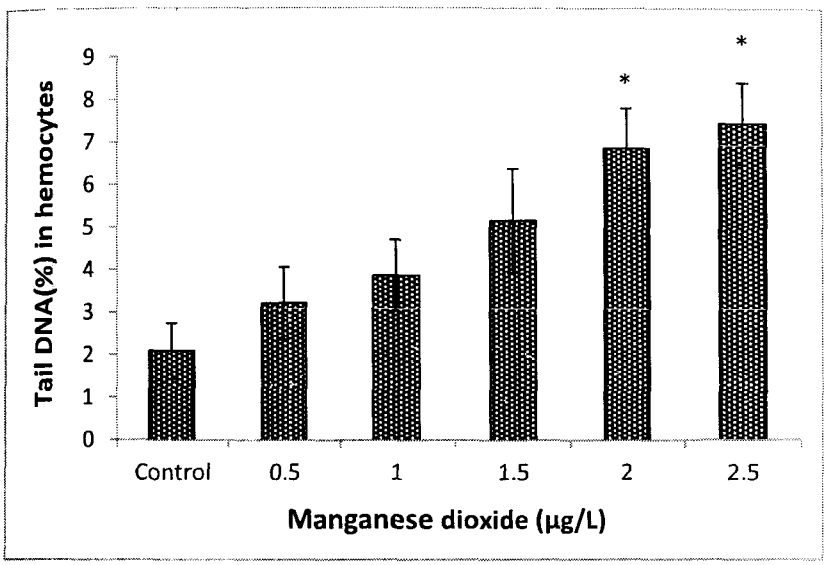
B



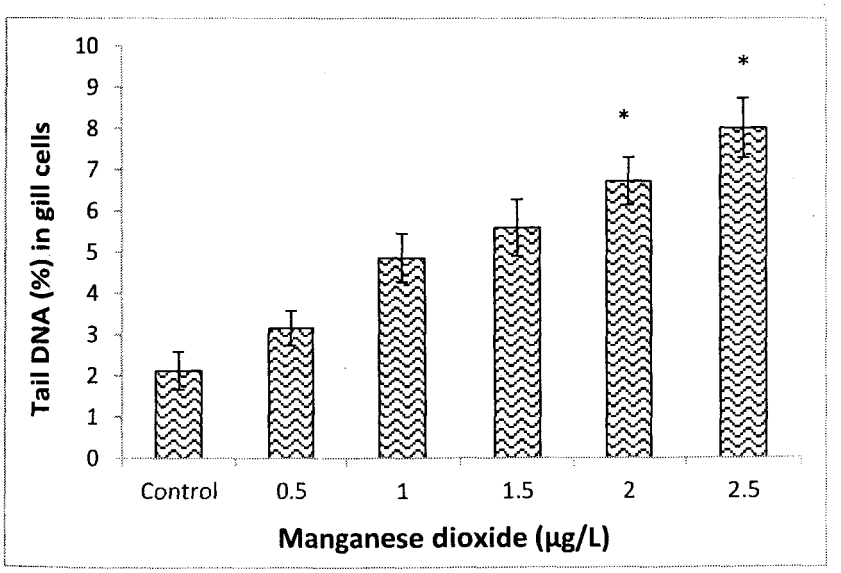
Note: * $p < 0.05$

Fig. 3.6. Tail DNA (%) in the **(A)** hemocytes and **(B)** gill cells of *M. casta* (Mean ± SD) exposed to various concentrations of manganese dioxide

A



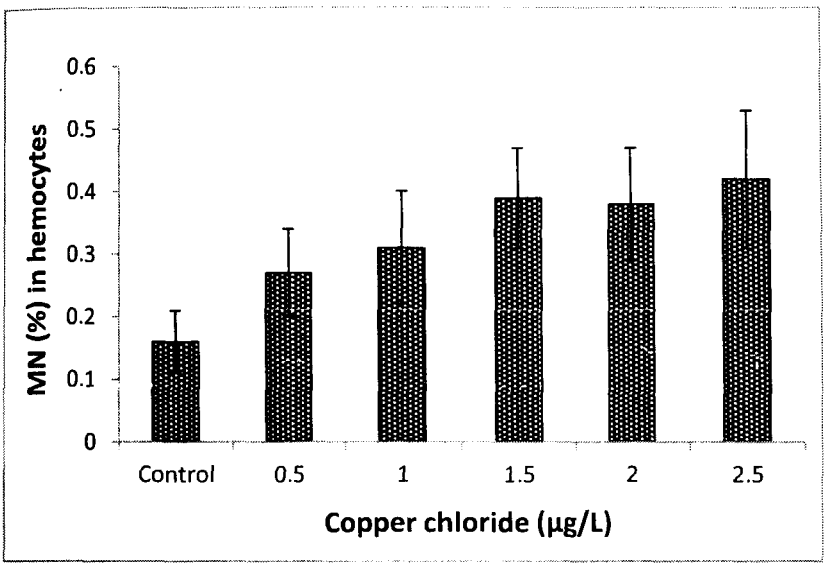
B



Note: * $p < 0.05$

Fig. 3.7. MN (%) in the **(A)** hemocytes and **(B)** gill cells of *M. casta* (Mean ± SD) exposed to various concentrations of copper chloride

A



B

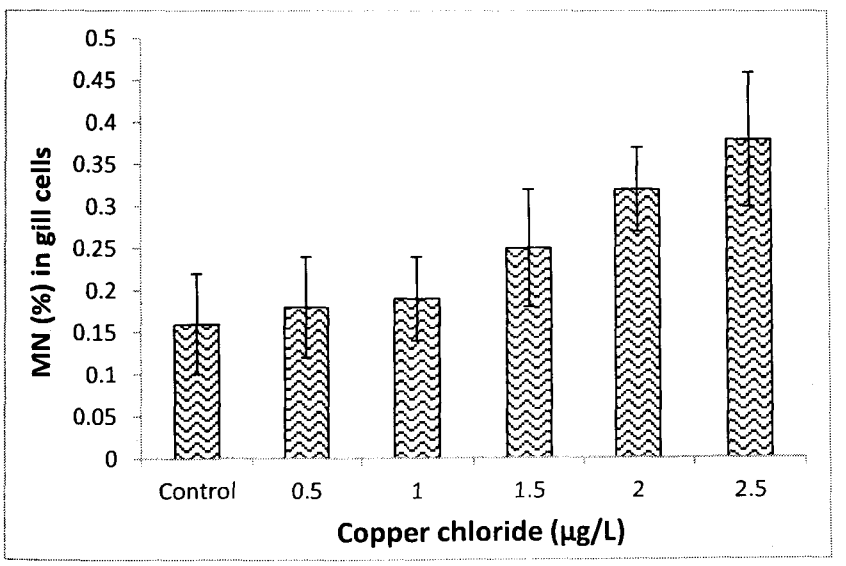
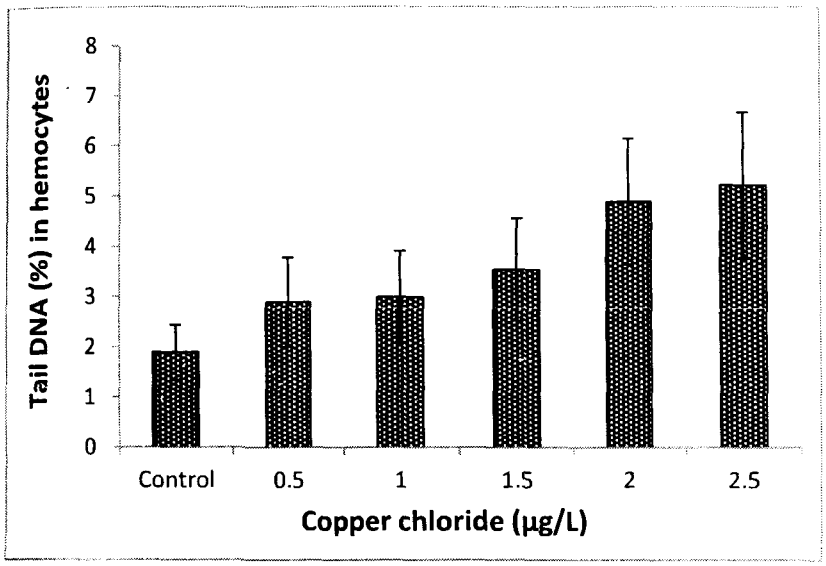


Fig. 3.8. Tail DNA (%) in the **(A)** hemocytes and **(B)** gill cells of *M. casta* (Mean ± SD) exposed to various concentrations of copper chloride

A



B

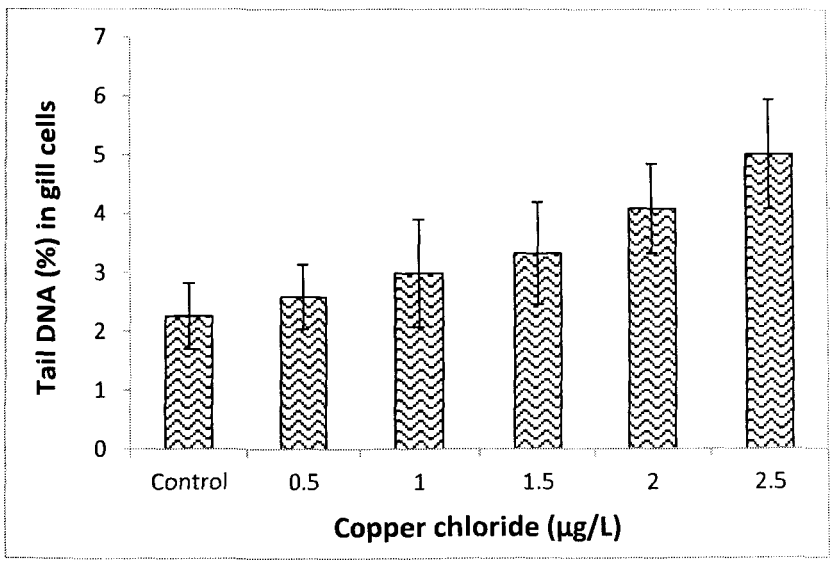
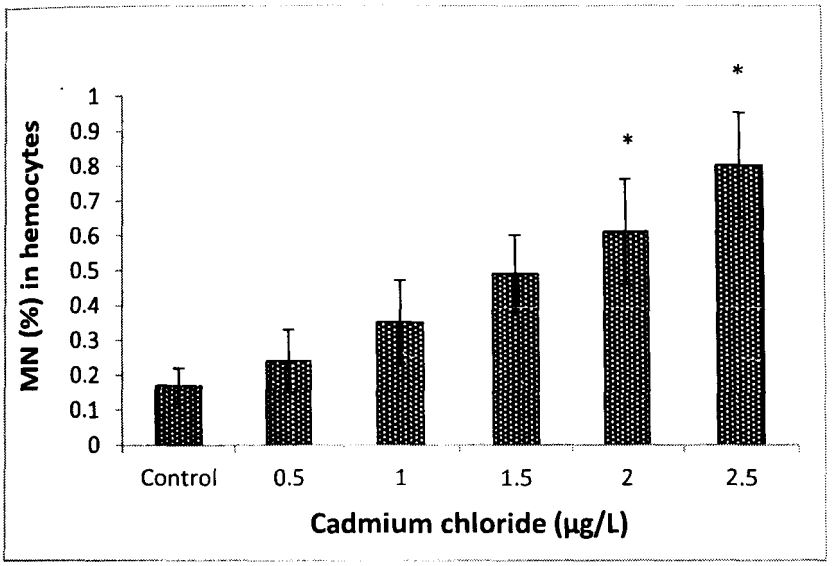


Fig. 3.9. MN (%) in the **(A)** hemocytes and **(B)** gill cells of *M. casta* (Mean ± SD) exposed to various concentrations of cadmium chloride

A



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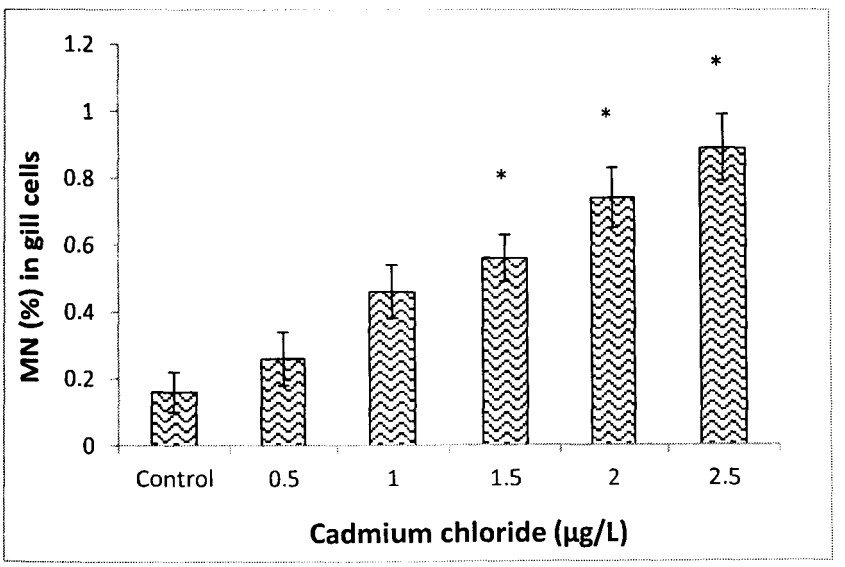
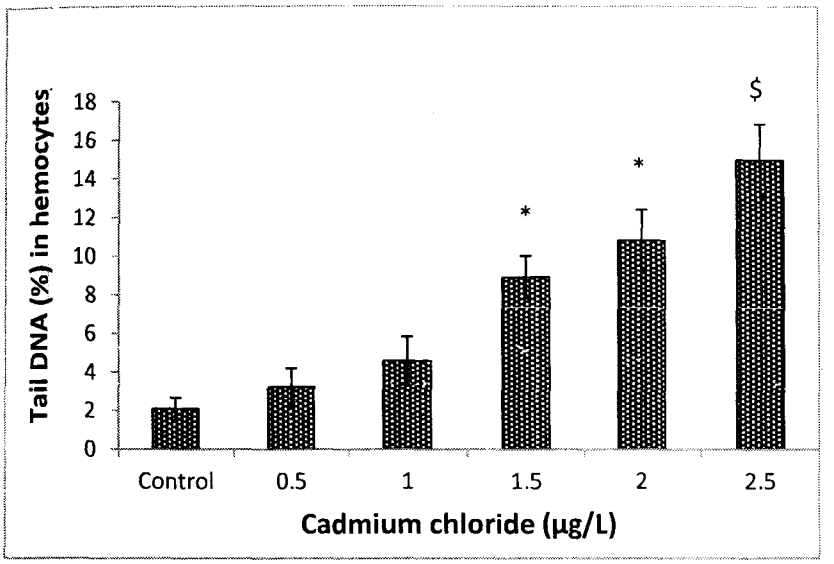


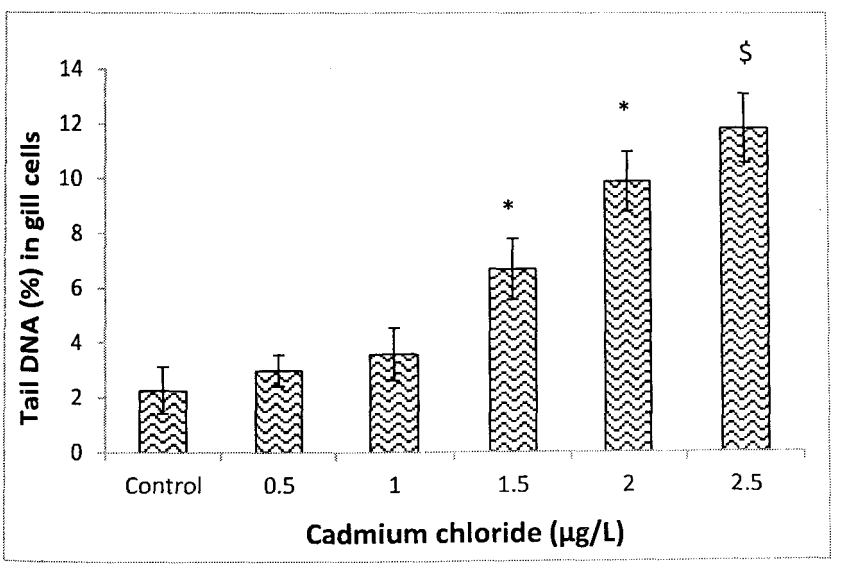
Note: * $p < 0.05$

Fig. 3.10. Tail DNA (%) in the **(A)** hemocytes and **(B)** gill cells of *M. casta* (Mean ± SD) exposed to various concentrations of cadmium chloride

A



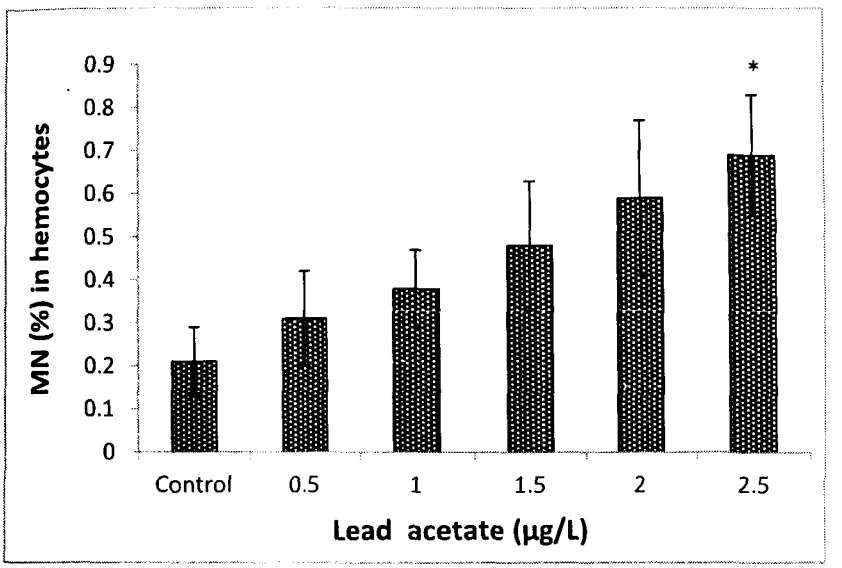
B



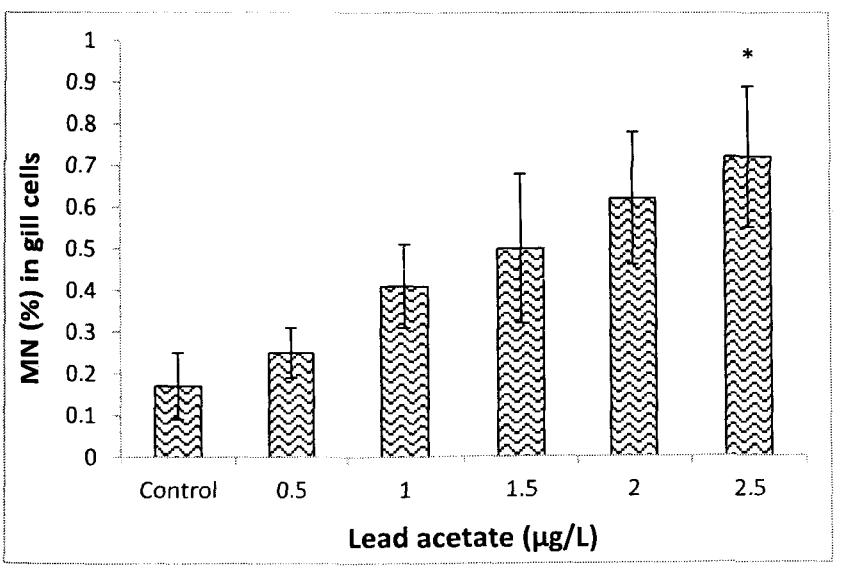
Note: * $p < 0.05$, $ $p < 0.01$

Fig. 3.11. MN (%) in the **(A)** hemocytes and **(B)** gill cells of *M. casta* (Mean ± SD) exposed to various concentrations of lead acetate

A



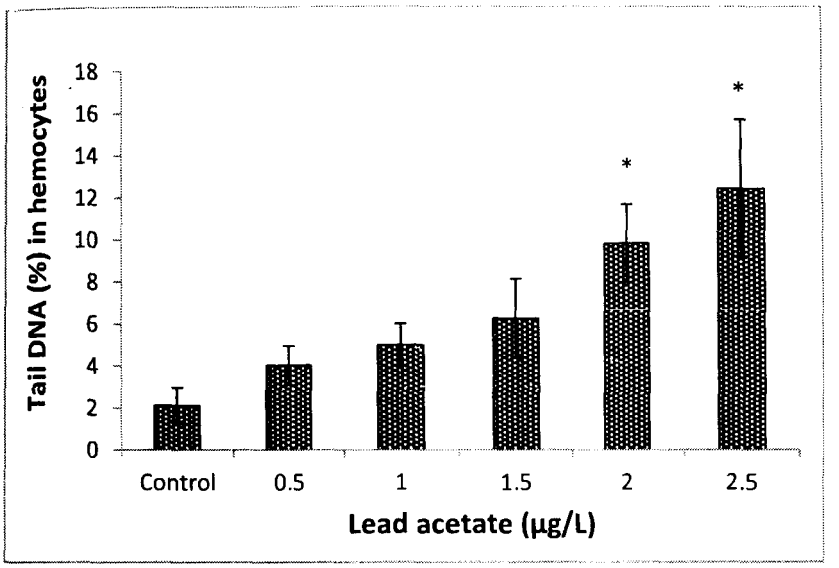
B



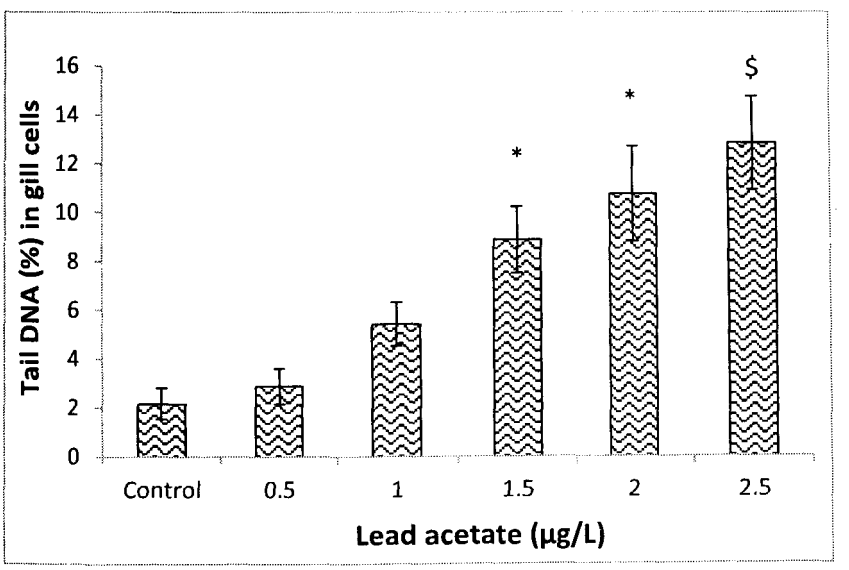
Note: * $p < 0.05$

Fig. 3.12. Tail DNA (%) in the **(A)** hemocytes and **(B)** gill cells of *M. casta* (Mean ± SD) exposed to various concentrations of lead acetate

A



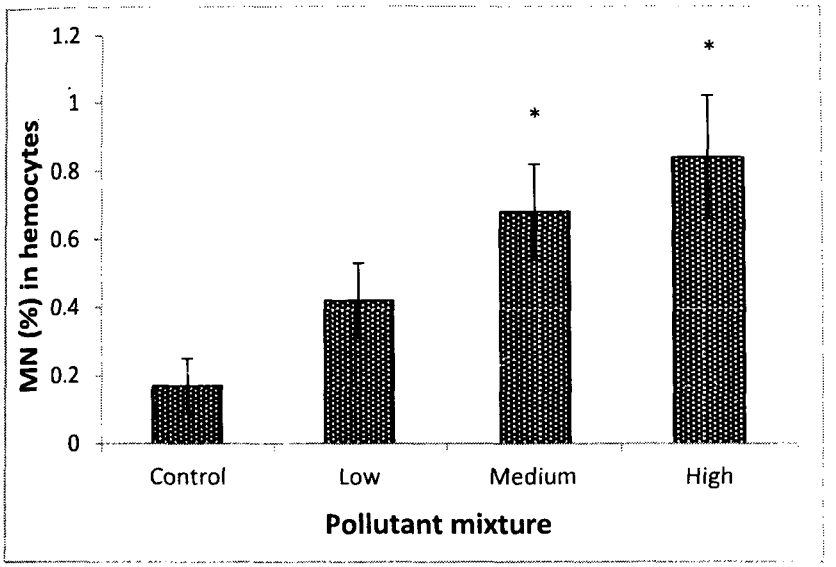
B



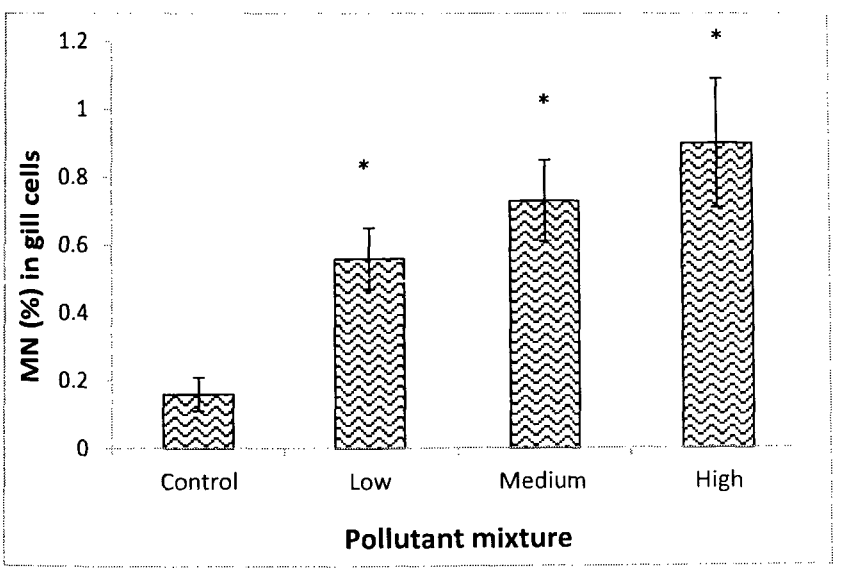
Note: * $p < 0.05$, $ $p < 0.01$

Fig. 3.13. MN (%) in the **(A)** hemocytes and **(B)** gill cells of *M. casta* (Mean ± SD) exposed to various concentrations of pollutant mixture

A

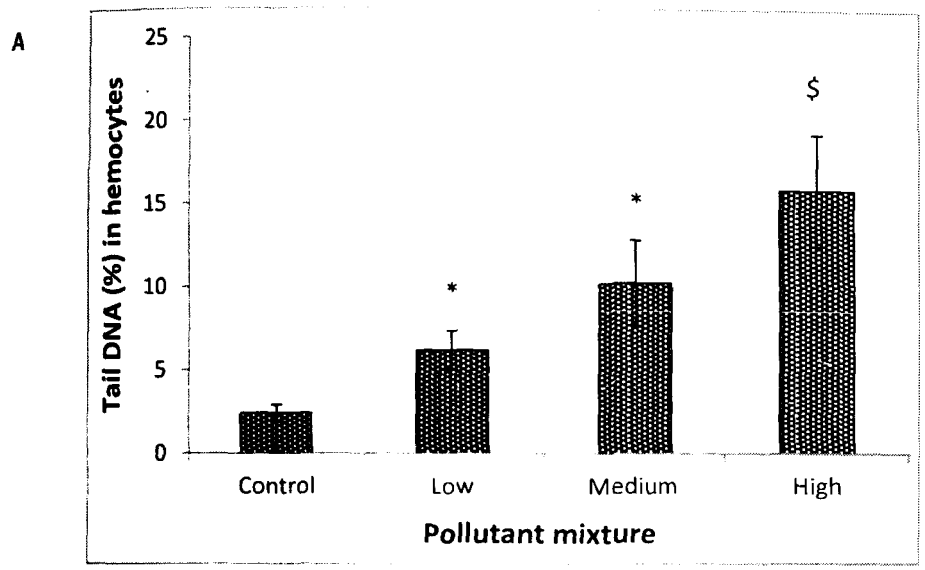


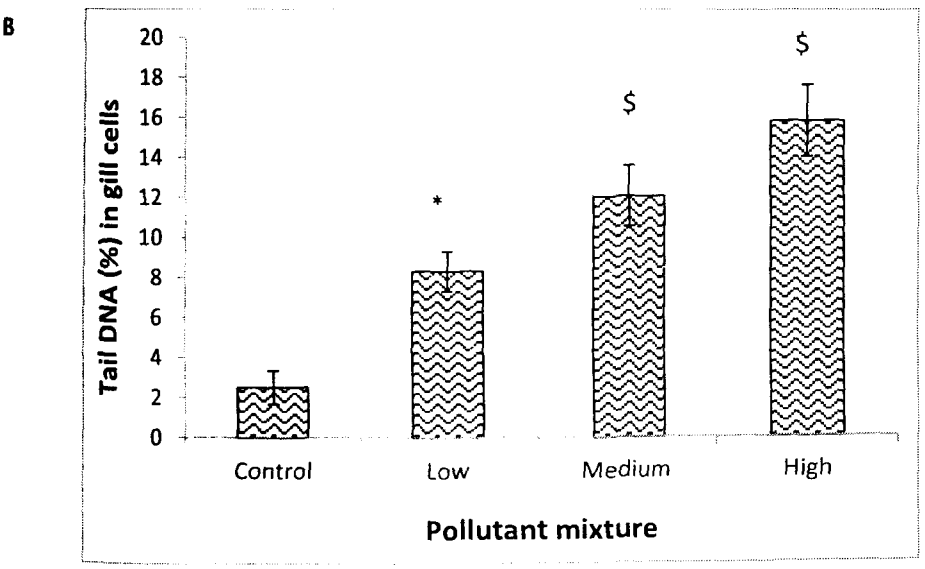
B



Note: * $p < 0.05$

Fig. 3.14. Tail DNA (%) in the **(A)** hemocytes and **(B)** gill cells of *M. casta* (Mean ± SD) exposed to various concentrations of pollutant mixture





Note: * $p < 0.05$, $ $p < 0.01$

3.5. Discussion

In the present study, total petroleum hydrocarbons (TPHs), Fe, Mn, Cd and Pb were found to induce significant DNA damage at the high doses studied in the hemocytes and gill cells of *M. casta*. However, Cu did not induce significant DNA damage at any of the concentrations studied.

The TPHs induced significant DNA damage as % tail DNA in the hemocytes as well as the gill cells of the bivalves at concentrations as low as 10 µg/L. This is in agreement with the findings of Pérez-Cadahía *et al.* (2004) in which mussels (*Mytilus galloprovincialis*) exposed to crude oil for 12 days exhibited significant DNA damage as measured by the comet assay. Further, TPHs induced significant MN at 15 µg/L in the hemocytes and 20 - 25 µg/L in the gill cells of *M. casta*. These results also find similarity with the report of Baršienė and Andreikėnaitė (2007) wherein MN were induced in the gill cells of mussels (*Mytilus edulis*) exposed to various concentrations of crude oil for 3 weeks. The PAH component of TPHs may be majorly responsible for causing DNA lesions. These PAHs when metabolized, get converted to diol-epoxides and then bind covalently to DNA leading to the formation of bulky adducts which are very difficult to repair. As a result, there will be accumulation of mutations which may induce carcinogenesis (Muñoz and Albores, 2011). PAHs are also known to induce the production of ROS which get converted to hydroxyl radicals and in turn damage DNA (Muñoz and Albores, 2011).

Bivalves exposed to high concentrations (10 µg/L) of Fe exhibited significant DNA damage as represented by the % of MN in the gill cells as well as the % tail DNA in both the gill cells and hemocytes. These findings are similar to those of Lemiere *et al.* (2005) in which Fe^{3+} (ferric ion) exposed to bivalves (*Unio tumidus*) induced significant DNA damage in their gill

cells. Iron induces DNA damage by either directly attacking the bases at specific sites (Lloyd and Phillips, 1999) or indirectly by the production of the hydroxyl radical which then damages the DNA (Valko et al., 2004). Iron also induces DNA lesions by the formation of adducts such as 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodGuo) which further leads to transversion mutations (Cooke *et al.*, 2003; Valko *et al.*, 2004; Lemiere *et al.*, 2005).

Very limited reports are available on the genotoxicity of Mn in spite of its importance as an essential trace element. However in the present study, we observed significant DNA damage at the higher concentrations of Mn. Few reports infer that Mn acts on DNA indirectly by the formation of oxygen radicals which could in turn damage DNA to some extent (Van Remmen *et al.*, 2003; Stephenson *et al.*, 2013).

Significant concentration-dependent increase of DNA damage as represented in both MN and % tail DNA was observed in the bivalves exposed to Cd. These observations are on par with those of Sarkar et al. (2013) in which the marine gastropod *Nerita chamaeleon* exhibited significant DNA damage when exposed to cadmium. Our results are also in agreement with that of Vincent-Hubert et al. (2011) wherein DNA damage was significant in the gill cells of *Dreissena polymorpha* exposed to cadmium over a 11-day period. DNA damage by Cd is known to occur via indirect mechanisms such as by the formation of ROS (Waalkes, 2000; Waisberg *et al.*, 2003; Bertin and Averbeck, 2006). In addition to DNA damage, Cd also interferes with the action of various proteins involved in the DNA repair process which could enhance and prolong the genotoxic condition (Waisberg *et al.*, 2003).

In the present study, Pb induced a significant increase of DNA damage in *M. casta* at the high doses. Black *et al.* (1996) also observed significant DNA damage in mussels (*Anodonta*

grandis) exposed to Pb. Lead may also affect DNA damage predominantly by indirect mechanisms of ROS formation and thereby leading to oxidative stress (Valverde *et al.*, 2001). Additional mechanisms of Pb-mediated genotoxicity also include inhibition of DNA synthesis, inhibition of DNA repair processes and also interaction with DNA-binding proteins and tumour suppressor proteins (Tchounwou *et al.*, 2012).

In contrast, to our laboratory observations of TPH and heavy metal induced genotoxicity, bivalves exposed to various concentrations of Cu did not induce significant DNA damage. Further, this observation also contradicts a few reports available on the genotoxicity of Cu in bivalves (Gabbianelli *et al.*, 2003; Villela *et al.*, 2006; Al-Subiai *et al.*, 2011). This could be due to the low concentration of Cu used in the present study (Cu concentration in the range of 0.5 µg/L to 2.5 µg/L) which is unable to induce DNA damage compared to the relatively higher concentrations used in the previous aforementioned reports (Cu concentration in the range of 18 µg/L to 1 mg/L). Thus, Cu may not have any direct effects in *M. casta* but may probably work synergistically with other metals in the aquatic environment.

The combination of all these pollutants was also able to induce DNA damage in the cells of *M. casta* to a significant extent. These experiments were done to know the extent of DNA damage in these organisms exposed to environmentally realistic concentrations of these pollutants. Our results are on par with that of Vincent-Hubert *et al.* (2011) in which they observed genotoxicity as MN and % tail DNA in the hemocytes and gill cells of the zebra mussel (*Dreissena polymorpha*) exposed to a combination of cadmium and benzo[a]pyrene. In another similar study, Andriėkenaitė *et al.* (2007) reported DNA damage as MN in the peripheral blood of rainbow trout (*Oncorhynchus mykiss*) exposed to a mixture of heavy metals which included Cu, Pb and Mn. DNA damage was also reported in the peripheral

blood of the Senegalese sole (*Solea senegalensis*) exposed to sediments contaminated with metals (such as Cu, Cd and Pb) and PAHs under laboratory conditions (Costa *et al.*, 2008). Co-exposure of organisms to both metals and petroleum hydrocarbons may lead to additive or non-additive (synergistic or antagonistic) toxic effects. Non-additive effects are usually implicated in ecotoxicological risk assessment because the toxicity of contaminant mixture is usually higher, in case of synergistic effects, than the summed toxicity of individual contaminants (Gauthier *et al.*, 2015). These toxic effects can arise from the interactions between the co-occurring toxicants, or through the effects of one toxicant on various processes involved in the transport, metabolism, and detoxification of the co-occurring toxicant.

3.6. Conclusions

Environmentally relevant concentrations of TPHs and heavy metals, particularly Fe, Mn, Cd and Pb are able to induce significant DNA damage in the form of MN and % tail DNA in the hemocytes as well as the gill cells of *M. casta*. Cu although unable to induce significant DNA damage at the test concentrations could be able to induce damage at higher concentrations. Further, DNA damage induced by a mixture of all these pollutants was found to be significant at almost all the concentrations. These pollutants could act antagonistically or synergistically in the environment or within the organism to induce additive or non-additive toxic effects.

Chapter 4:

Biochemical and physiological tests as biomarkers of genotoxicity

4.1. Introduction

The genotoxicity of pollutants in aquatic organisms may be due to their direct effects on the genome where the pollutant molecules directly interact with the nucleic acids or indirectly by interacting with other molecules such as water to give rise to highly reactive molecules. These molecules are called reactive oxygen species (ROS) and are implicated in the cellular oxidative stress within organisms. These ROS can be produced under natural conditions by the normal basal metabolism or by the influence of environmental factors. Naturally produced ROS can easily be reduced by antioxidants that are produced by the body. However, ROS production mediated by environmental stressors build up to much higher levels and they can overcome the antioxidant defenses. Oxidative stress is therefore considered to be an imbalance between oxidants and antioxidants at the cellular level. Oxidative damage as a result of such an imbalance may cause oxidative modification of cellular macromolecules such as the induction of cell death by apoptosis or necrosis, as well as structural tissue damage. As a result, these ROS can affect various cellular processes and organelles. DNA is one of the key cellular components that are highly susceptible to the action of ROS. Aquatic organisms can provide model systems for investigation of how cells respond to damage by ROS, how repair mechanisms can bring about amelioration of this damage and how oxidative stress can subsequently cause other deleterious effects and disease (Livingstone *et al.*, 1994).

ROS include the hydroxyl radical ($^{\bullet}OH$), superoxide anion radical ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2). These chemical species are highly reactive and react with DNA, lipids, proteins and carbohydrates, often leading to destructive effects (Storey, 1996). These ROS must therefore be eliminated or converted to less harmful substances by antioxidants. Among

the antioxidants, catalase is one of the major enzymes that decomposes H_2O_2 to less-reactive molecular oxygen and water and thereby protecting cells from oxidative damage. The heterogeneous structure of DNA molecules including the bases and the sugar–phosphate backbone is ideal for attack by $^{\cdot}OH$ (Buxton *et al.*, 1988). These hydroxyl radicals react with bases with a higher affinity than with the nucleic acid backbone (Cadet *et al.*, 1997). More than 100 different products of DNA damage are known to be formed as a result of attack by ROS (Dizdaroglu, 1993). One of the most important DNA lesion that is also used as a biomarker for cellular oxidative stress and genotoxicity in living organisms is 8-hydroxy-20 - deoxyguanosine (8-OHdG) or its oxidation product 8-oxo-20 –deoxyguanosine (8-oxodG). Transition metals play an important role in these oxidative reactions (Nackerdien *et al.*, 1991). Hydroxyl radical attack on the sugar–phosphate backbone of DNA can cause different lesions such as apurinic sites where the base has been removed, fragmentation of deoxyribose causing single-strand breaks and oxidation of the sugar moiety (Dizdaroglu *et al.*, 1975; Breen and Murphy, 1995; Valavanidis *et al.*, 2006).

Lipids in cell membranes are continuously exposed to oxidants from endogenous and exogenous sources and may result in a threat to cells. This leads to lipid peroxidation which involves the formation and propagation of lipid radicals, rearrangement of the double bonds in unsaturated fatty acids which ultimately leads to the destruction of membrane lipids and the production of a variety of breakdown products such as ketones, aldehydes, alcohols, ethers and alkanes (Dianzani and Barrera, 2008; Repetto *et al.*, 2012). Malondialdehyde (MDA) is a product of lipid (polyunsaturated fatty acids) peroxidation in cells caused a result of ROS. MDA in turn reacts with the nucleotides in DNA leading to formation of DNA

adducts (Marnett, 1999). MDA is therefore potentially mutagenic and its presence often indicative of oxidative stress.

Acetylcholinesterase (AChE), the primary cholinesterase in organisms, catalyzes the breakdown of acetylcholine. It is found mainly at neuromuscular junctions and chemical synapses where it terminates synaptic transmission. The enzyme can be inactivated by various chemicals which subsequently leads to acetylcholine accumulation, hyperstimulation of muscarinic and nicotinic receptors and disrupted neurotransmission. This AChE inhibition can therefore result in overstimulation of the central and peripheral nervous systems, resulting in neurotoxic effects, behavioural changes and ultimately death (Xuereb *et al.*, 2009). A number of chemicals such as Cd are known to interfere with AChE activity which may be mediated via ROS (Wang and Wang, 2009). Meaurement of AChE activity can be considered to be a useful biomarker for evaluating the effects of exposure to neurotoxic substances in the aquatic environment (Cajaraville *et al.*, 2000; Matozzo *et al.*, 2005).

The condition index is a measurement of the overall health of an organism by comparing various physical characteristics to give a ratio (eg: shell weight, length, soft tissue weight, etc). Significant variations in meat content may occur in bivalves depending upon the physiological condition and variation in environmental parameters such as availability of metals and petroleum hydrocarbons. Effects in DNA may also cause altered protein production which may result in decreased health of the organism. The condition index is therefore a simple measurement that can be used to provide information about the "well-being" of the organism (Mouneyrac *et al.*, 2008).

4.2. Review of Literature

A number of antioxidant enzymes and physiological tests are used in bioindicator organisms to evaluate the effects of contaminants in water bodies. Kamel *et al.* (2014) used a number of biomarkers (catalase, malondialdehyde, acetylcholinesterase, benzo[a]pyrene hydroxylase, glutathione-S-transferase and metallothioneins) to understand the integrated biological response of the mussel, *Mytilus galloprovincialis* collected from Bizerte lagoon, which is a region highly polluted with heavy metals and petroleum hydrocarbons in Tunisia. In another study, Leiniö and Lehtonen (2005) analyzed the AChE, CAT, glutathione-S-transferase and metallothionein activities in two bivalve species, *Mytilus edulis* and *Macoma balthica* from inshore and offshore sites in the Baltic Sea. These multi-biomarker approaches therefore give a broader perspective of the variable effects of environmental contaminants in the fauna inhabiting these waters.

Boudjema *et al.* (2014) reported a significant increase in CAT activity in the tissues of brown mussels (*Perna perna*) that were exposed to environmentally relevant concentrations of cadmium, lead and copper. Additionally, increasing CAT activity was found to be correlated with increasing concentrations of these metals. An increase in the CAT activity was observed by Jebali *et al.* (2007) in the soft tissues of the clam *Ruditapes decussatus* collected from polluted sites along the coast of Tunisia. The CAT activity was also found to be significantly correlated with oxidative DNA damage in these clams. Significantly high CAT activity was also reported in bivalves, *Scrobicularia plana* and *Cerastoderma edule* from an estuary polluted with sewage discharges and industrial effluents in Morocco (Bergayou *et al.*, 2009). In another biomonitoring study, the CAT activity was found to be significantly higher in the tissues of the mussel *Mytilus galloprovincialis* collected from a coastal area polluted with

heavy metals compared to that collected from an unpolluted site from Greece (Vlahogianni *et al.* 2007).

Oxidative stress quantified by the malondialdehyde activity in the bivalve *Modiolus modiolus* was found to increase as a consequence of exposure to Cd under laboratory conditions (Dovzhenko *et al.* 2005). Lipid peroxidation was also reported by Wadige *et al.* (2017) in the freshwater bivalve *Hyridella australis* that were exposed to sediments contaminated with metals such as Cd and Zn. Sureda *et al.* (2013) analyzed the oxidative stress induced by petroleum hydrocarbon in an endemic bivalve *Pinna nobilis* as a result of exposure to an oil spill in the Mediterranean Sea and reported significant increases in the CAT and MDA activity compared to bivalves collected from an unpolluted area. Romero-Ruiz *et al.* (2003) reported an increase in MDA levels in the gills of *S. plana* transplanted to an estuary in Spain contaminated with heavy metals such as Cd, Fe, Mn, and Pb due to mining activities. They also suggested that these metals exerted oxidative stress in these bivalves which could lead to DNA damage. Similarly, in another study, the activities of CAT and MDA were found to be highly significant in *Mytilus galloprovincialis* at a metal-polluted site on along the coast of Spain (Funes *et al.*, 2005). Zanette *et al.* (2011) reported an increase in MDA levels of the oyster *Crassostrea gigas* exposed to different concentrations of diesel oil. They also observed a positive correlation between the salinity and MDA levels indicating that salinity conditions may influence pollutant uptake. Lipid peroxidation was also found to be significantly increased in the tissues of the scallop *Pecten maximus* exposed to phenanthrene which is also a toxic component of crude oil (Hannam *et al.*, 2010).

Acetylcholinesterase (AChE) inhibition is a well-established biomarker of neurotoxic effects and commonly used in aquatic biomonitoring. The seasonal variations in acetylcholinesterase

(AChE) activity in marine snails (*Cronia contracta*) along the coast of Goa provide an insight into the extent of pollution by neurotoxic substances (Gaitonde *et al.*, 2006). *In vitro* studies on acetylcholinesterase inhibition by toxic heavy metals (Pb, Cd, and Cu) and petroleum hydrocarbons clearly differentiate the neurotoxic potentials of various contaminants in coastal environment. The antioxidant enzyme CAT and the neurotoxicity biomarkers AChE were used as biomarkers to assess heavy metal and petroleum contamination in the clam *S. plana* collected from a marsh ecosystem in Spain (Solé *et al.*, 2009). Machreki-Ajmi and Hamza-Chaffai (2008) reported a significant inhibition of AChE activity and an increase in MDA activity in cockles (*Cerastoderma glaucum*) transplanted from an unpolluted site to a site contaminated with Cd in Tunisia. Mussels (*Mytilus galloprovincialis*) exposed to various concentrations of benzo[a]pyrene showed an increase in CAT activity and a subsequent depression of AChE activity as the exposure time increased (Banni *et al.*, 2010). Clams (*Ruditapes decussatus*) and mussels (*Mytilus galloprovincialis*) collected from sites polluted with heavy metals exhibited decreased acetylcholinesterase activity compared to those collected from unpolluted sites in Tunisia (Dellali *et al.*, 2001). Mussels (*Perna viridis*) transplanted to sites polluted with petroleum hydrocarbons in Hong Kong exhibited significant CAT and lipid peroxidation in their soft tissues (Cheung *et al.*, 2001). Further, these biomarkers were found to be correlated with concentration of PAHs in their soft tissues indicating their genotoxic potential. AChE assay was also used as a biomarker to test the presence of neurotoxic compounds in the clam *Tapes philippinarum* (Matozzo *et al.*, 2005) and *Mytilus galloprovincialis* (Lionetto *et al.*, 2003) collected from sites polluted with persistent pollutants in Italy.

The condition index, which is a measure of physiological well-being of an organism has also been used to assess the health of bivalves (Orban *et al.*, 2002; Marin *et al.*, 2003; Filgueira *et al.*, 2013). A significant reduction in the condition index (CI) of the bivalve *Anadara trapezia* experimentally exposed to sediments contaminated with heavy metals such as Cd, Cu and Pb was reported by Taylor and Maher (2015).

4.3. Materials and Methods

4.3.1. Quality assurance and quality control

The appropriate quality assurance methods of sample preparation, handling and preservation were carried out in accordance with US EPA procedures. All chemicals used were of analytical grade from Himedia (Himedia, India) unless specified otherwise.

4.3.2. Maintenance of *Meretrix casta*

Meretrix casta were maintained as detailed in chapter 3 (section 3.3.2)

4.3.3. Treatment schedule

The treatment schedule is outlined in chapter 3 (section 3.3.3)

4.3.4. Catalase assay

Bivalves were dissected and their whole soft body tissues were collected and homogenized in 50 mM of Tris buffer (pH 7.4) containing 0.3M sucrose and 1mM EDTA. This suspension was then centrifuged at 10,000xg for 20 min at 4°C and the supernatant was collected. Catalase activity was carried as per Aebi (1984) based on the decrease in absorbance of the test sample by the decomposition of H_2O_2. The reaction mixture consisted of 13.2 mM H_2O_2 in 50 mM phosphate buffer (pH 7.0) and 0.1 ml of the homogenate. The reduction in absorbance was measured at 240 nm using a multiwall plate reader (Analytical Technologies

Ltd.) at 25°C over 3 minutes. Total protein concentration was measured by Bradford's method (Bradford, 1976). The activity of catalase (CAT) was expressed as μmol $H_2O_2^{-1}min^{-1}$mg protein.

4.3.5. Acetylcholinesterase assay

The acetylcholinesterase (AChE) activity in whole soft body tissues of bivalves was determined using the protocol of Ellman *et al.* (1961) with modifications as described by Galloway *et al.* (2002). Briefly, 50 μL of sample homogenate was incubated in microtitre plates with 150μl DTNB (270μM in 50mM sodium phosphate pH 7.4) at 25°C for 5 min. The enzyme activity was initiated by the addition of 3 mM acetylthiocholine iodide and the absorbance was measured at 412 nm. The activity of AChE was expressed as nmol $thiocholine^{-1}min^{-1}$mg protein.

4.3.6. Malondialdehyde assay

The malondialdehyde (MDA) assay which is used to test lipid peroxidation in the whole soft body tissues of bivalves was carried out using a commercial kit (North West Life Science Specialities- NWK-MDA01). The assay is based on the reaction of MDA with thiobarbituric acid (TBA) forming a pink coloured MDA-TBA_2 adduct that absorbs strongly at 532 nm. Butylated hydroxytoluene (BHT) and EDTA are also added to the reaction mixture containing the sample homogenate to minimize oxidation of lipids. The activity of MDA was expressed as nmol $MDA^{1}min^{-1}$mg protein.

4.3.7. Condition index

Bivalves were cleaned, dissected and the soft tissues were carefully separated from the hard shells. Both the soft tissue and the shells were placed separately in an oven (REMI) at 60°C

overnight to determine their dry weights (Filgueira *et al.*, 2013). Condition index (CI) was then calculated as follows:

$$\text{Condition index} = \frac{\text{Dry soft tissue weight (g)}}{\text{Dry shell weight (g)}} \times 100$$

4.3.8. Statistical analyses

Statistical analyses of the data were carried out using IBM SPSS 23 statistical software package. The data were tested to meet the assumptions of normality and homogeneity prior to subsequent analyses by linear models. The data of the MN test and comet assay from the previous chapter are expressed as percentage values and were therefore arc sine transformed whereas the data of CAT, AChE and MDA assays were log transformed. A one-way ANOVA was applied to test the effect of treatment on the % MN, % Tail DNA, CAT, AChE, MDA and CI with a post hoc Dunnet's test to compare the different groups with the control within the same treatment group. Pearson's correlation was also used to test the relationship between the all the parameters. The data were considered to be statistically significant at $p < 0.05$.

4.4. Results

4.4.1. Catalase assay

The data on the CAT activity in the bivalves exposed to various concentrations of the pollutants are represented in figs. 4.1.A-G. In the present study, Cd, Pb, TPHs and the pollutant mixture were able to induce oxidative stress as observed by the levels of CAT in the soft tissues of *M. casta*. The CAT activity of bivalves exposed to Cd was significant ($p < 0.05$) at the 2 and 2.5 µg/L concentrations (fig. 4.3a). In case of Pb and TPHs, CAT activity

was significant ($p < 0.05$) at the highest dose of 2.5 and 25 µg/L respectively. The pollutant mixture was able to induce significant CAT activity ($p < 0.05$) at all the concentrations (4.1.G). None of the concentrations of Fe and Cu were able to induce a significant increase of CAT activity in the bivalves.

4.4.2. Malondialdehyde assay

Data on the levels of MDA in the soft tissues of *M. casta* are represented in figs. 4.2.A-G. The MDA content in the bivalves exposed to the highest concentration of Mn were found to be significant ($p < 0.05$). Cd was able to significantly elevate ($p < 0.05$) the levels of MDA in the soft tissues of the bivalves at the 2 and 2.5 µg/L concentration. The levels of MDA were also found to be significant in the Pb-exposed bivalves, especially at the 1.5, 2 and 2.5 µg/L concentrations. In the TPH-exposed bivalves, significance of MDA was observed only at the highest concentration of 25 µg/L. In case of the pollutant mixture, MDA levels were significant only at the highest concentration ($p < 0.05$). On the other hand, Fe and Cu were unable to induce any significant changes in the levels of MDA.

4.4.3. Acetylcholinesterase assay

The data on the AChE activity in the bivalves exposed to various concentrations of the pollutants are represented in figs. 4.3.A-G. The activity of AChE was found to decrease with decreasing concentrations of the pollutants particularly Mn, Cd, Pb, TPHs and the pollutant mixture. Significantly depressed AChE was observed only at the highest concentration (2.5 µg/L) of Mn. In case of the bivalves exposed to Cd, significant decrease of AChE activity was observed at the 2 and 2.5 µg/L concentrations, whereas that of the Pb-exposed group was significant at 1.5, 2 and 3.5 µg/L concentrations ($p < 0.05$). TPH-exposed bivalves exhibited significant depression of AChE activity at the 20 and 25 µg/L concentrations. In the pollutant

mixture group, AChE activity was found to be significantly decreased at the medium and high concentrations ($p < 0.05$). Additionally, AChE activities of the bivalves exposed to Fe and Cu did show any significant changes.

4.4.4. Condition index

The data on the CI of the bivalves exposed to various concentrations of the pollutants are represented in figs. 4.4.A-G. Significant depression of the CI was observed in the bivalves exposed to the highest concentration of Fe (10 µg/L; $p < 0.05$). The CI of the bivalves exposed to Mn, Cd and Pb was also found to be significantly decreased at the highest concentration of 2.5 µg/L ($p < 0.05$). In the TPH-exposed group, a significant decrease of the CI was observed at the highest concentration of 25 µg/L. In case of the pollutant mixture, significant decrease of the CI was observed at the medium and high concentrations ($p < 0.05$). The Cu-exposed group did not show any significant decrease of the CI ratio at any concentration studied.

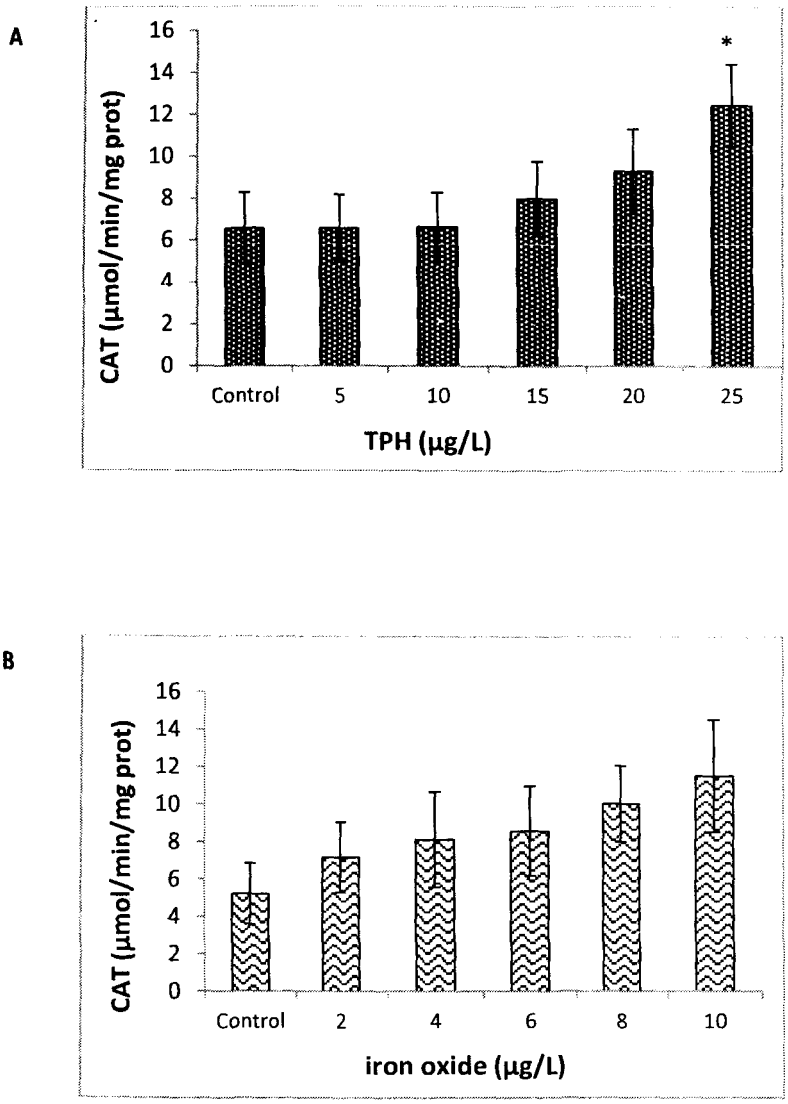
4.4.5. Correlation analyses

Correlation analyses were carried out between each pollutant treatment and the biomarker responses and are represented in tables 4.1.A-G. A positive correlation was observed between DNA damage parameters as well as all the biomarkers (Table 4.1a) in the bivalves exposed to TPHs. A high positive correlation was observed between % MN and CAT activity ($R = 0.90$, $p < 0.001$) and between % tail DNA and CAT activity ($R = 0.92$, $p < 0.001$). Similarly, a high positive correlation was also observed between both DNA damage parameters and MDA ($p < 0.001$). Significant negative correlations between oxidative stress parameters (CAT and MDA) and AChE were also observed ($R = -0.72$, $p < 0.01$ and $R = -0.85$, $p < 0.001$). Similarly, the CI was found to be negatively correlated with the DNA

damage parameters as well as with the oxidative stress parameters. In the Fe-exposed group however, correlations were only observed between DNA damage parameters and oxidative stress parameters (Table 4.1.B). No correlations were observed between AChE and DNA damage parameters and oxidative stress parameters. CI also did not correlate with any of the above parameters. In the Mn-exposed group, a significant positive correlation was observed between % MN and CAT ($R = 0.56$, $p < 0.05$) as well as between % tail DNA and CAT ($R = 0.59$, $p < 0.05$). Significant positive correlations were also observed between both DNA damage parameters and MDA. AChE values correlated with MDA values ($R = -0.76$, $p < 0.01$), however, no correlation was observed between AChE and CAT. CI also correlated significantly with all the parameters except with CAT activity.

Bivalves exposed to Cu did not display any significant correlation between any of the biomarkers measured in them (Table 4.1.D). The Cd-exposed group (Table 4.1.E) on the other hand exhibited significant correlations between all DNA damage parameters, oxidative stress parameters, AChE and CI ($p < 0.001$). A similar trend was also observed in the bivalves exposed to Pb (Table 4.1.F). DNA damage parameters correlated significantly with oxidative stress parameters ($p < 0.001$). Positive correlations were also observed between AChE and DNA damage parameters as well as with oxidative stress parameters. CI also correlated significantly with DNA damage parameters and oxidative stress parameters. Significant correlations were also observed between all the parameters in the bivalves exposed to the pollutant mixture. DNA damage parameters correlated significantly with the oxidative stress parameters ($p < 0.001$). AChE and CI also correlated significantly with oxidative stress parameters ($p < 0.001$).

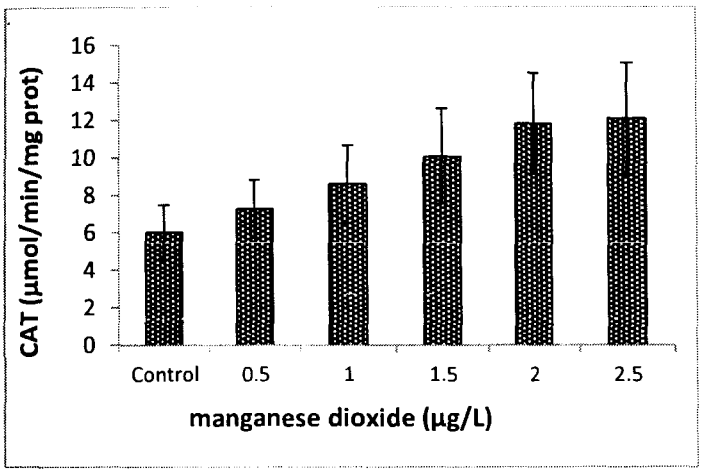
Fig. 4.1. Catalase (CAT) activity in the soft tissues of *M. casta* (Mean ± SD) exposed to various concentrations of **(A)** TPH and **(B)** iron oxide



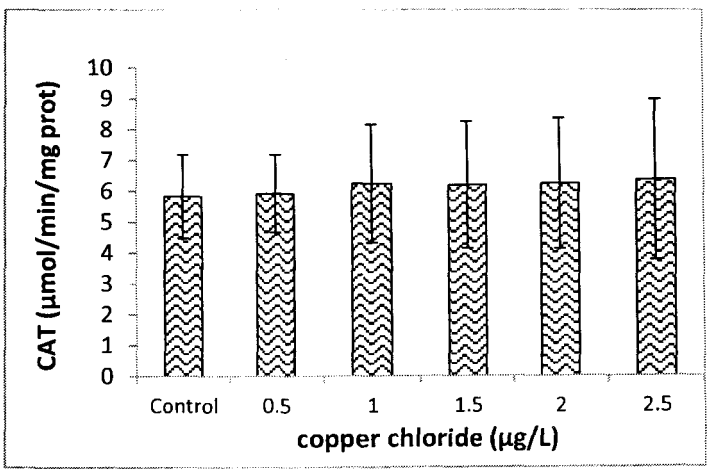
Note: * $p < 0.05$

Fig. 4.1. Catalase (CAT) activity in the soft tissues of *M. casta* (Mean ± SD) exposed to various concentrations of **(C)** manganese dioxide and **(D)** copper chloride

C

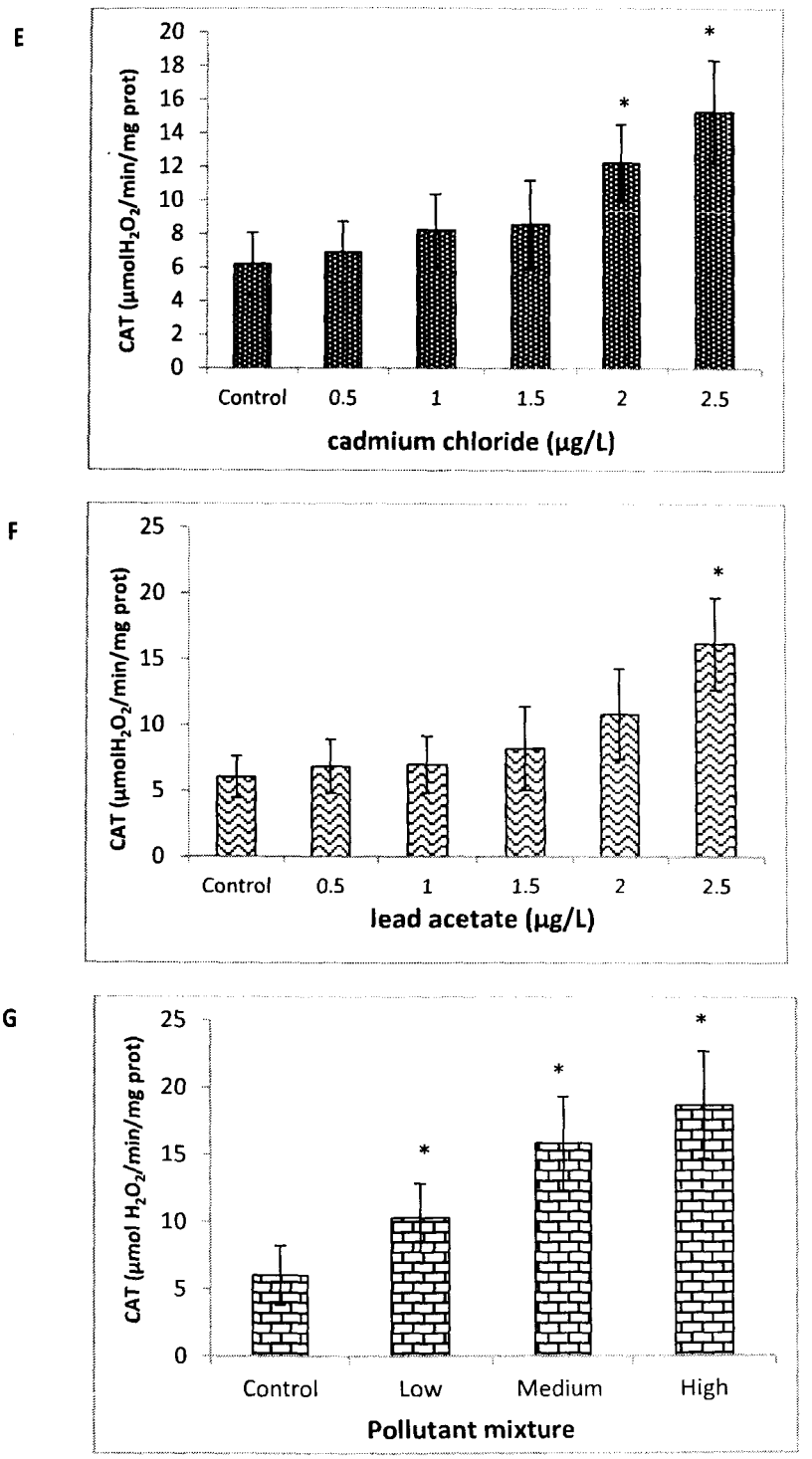


D



Note: * $p < 0.05$

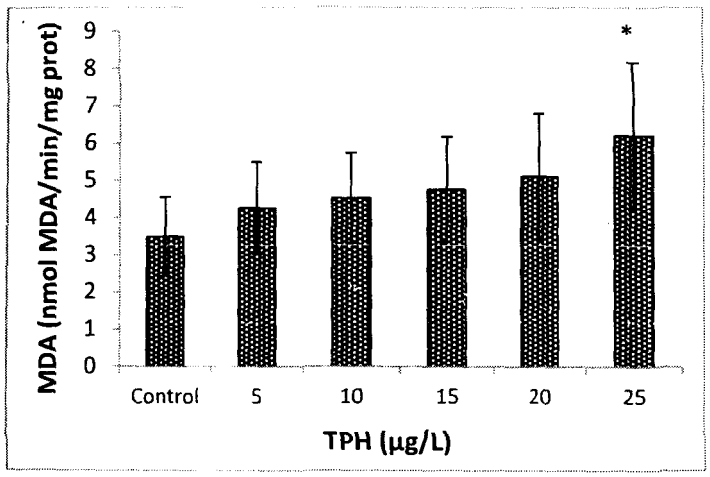
Fig. 4.1. Catalase (CAT) activity in the soft tissues of *M. casta* (Mean ± SD) exposed to various concentrations of **(E)** cadmium chloride **(F)** lead acetate and **(G)** pollutant mixture



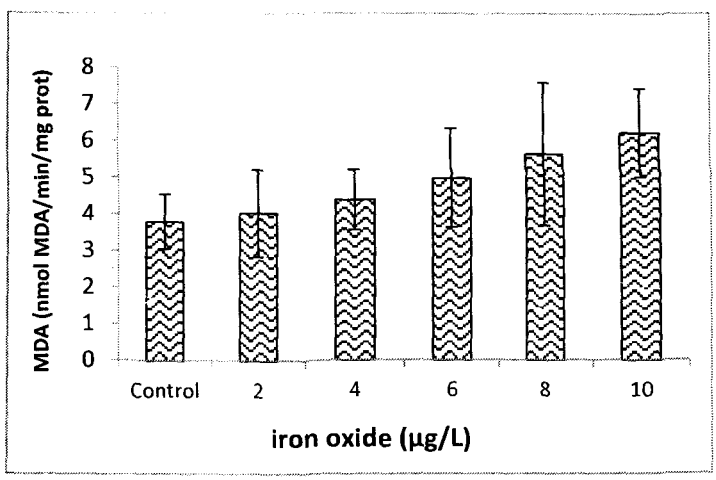
Note: * $p < 0.05$

Fig. 4.2. Malondialdehyde (MDA) in the soft tissues of *M. casta* (Mean ± SD) exposed to various concentration of **(A)** TPH and **(B)** iron oxide

A



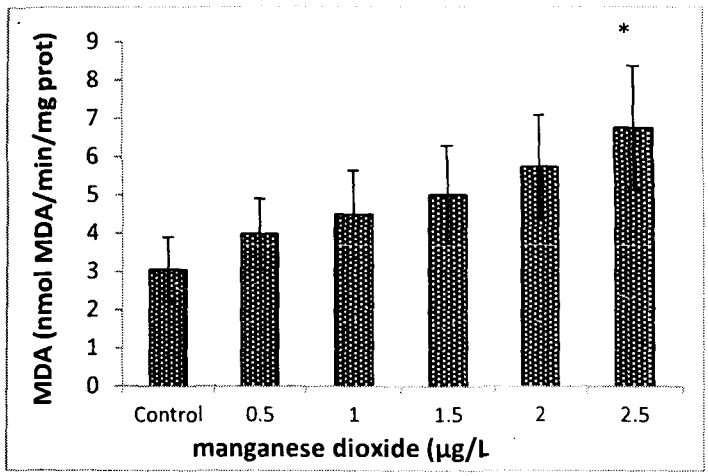
B



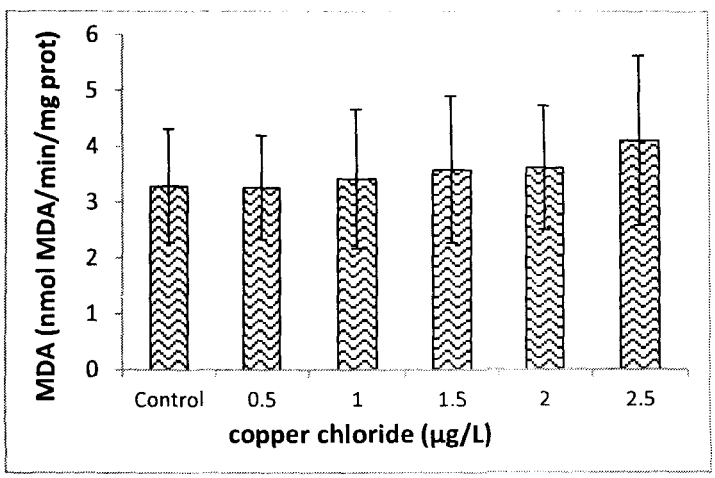
Note: * $p < 0.05$

Fig. 4.2. Malondialdehyde (MDA) in the soft tissues of *M. casta* (Mean ± SD) exposed to various concentration of **(C)** manganese dioxide and **(D)** copper chloride

C

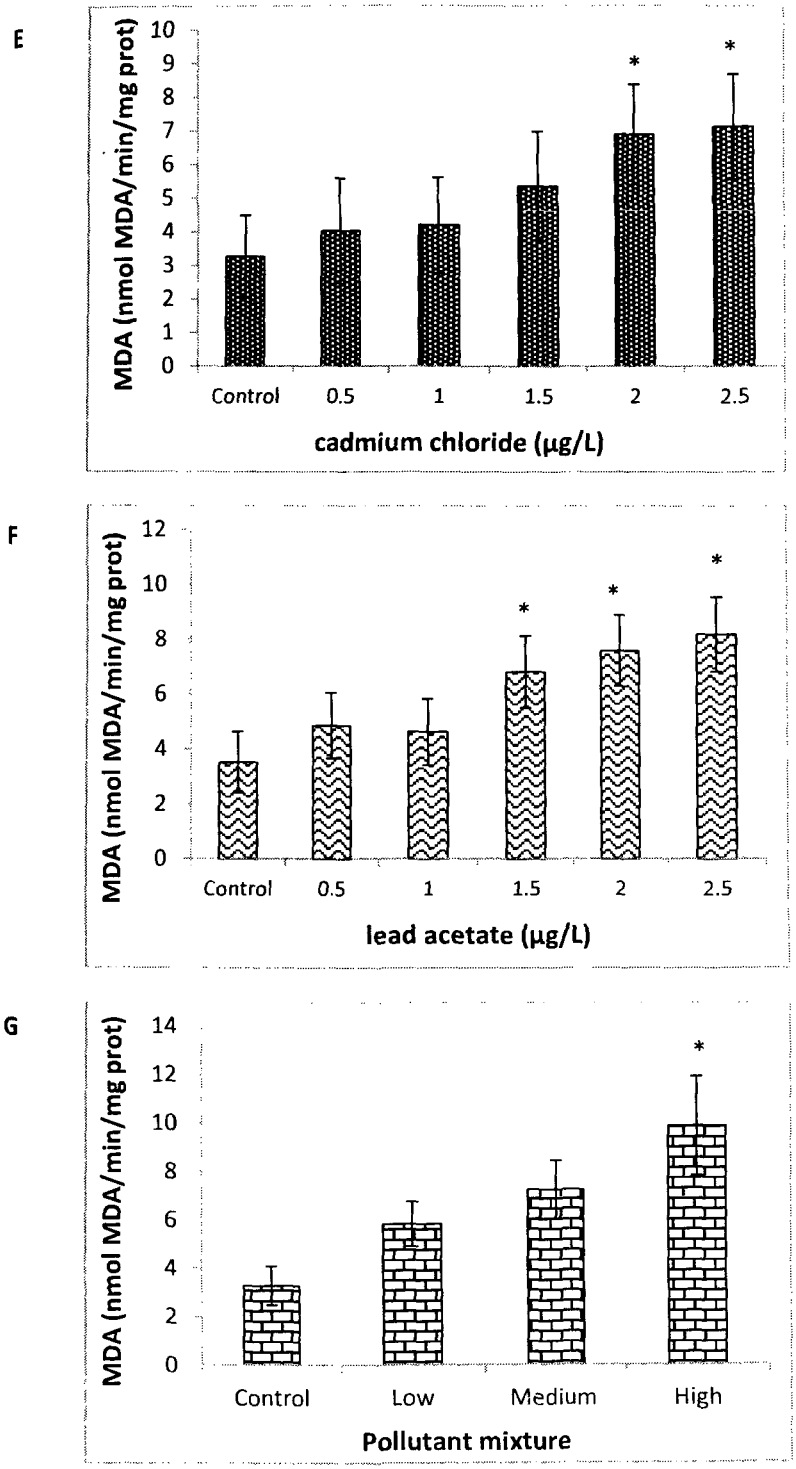


D



Note: * $p < 0.05$

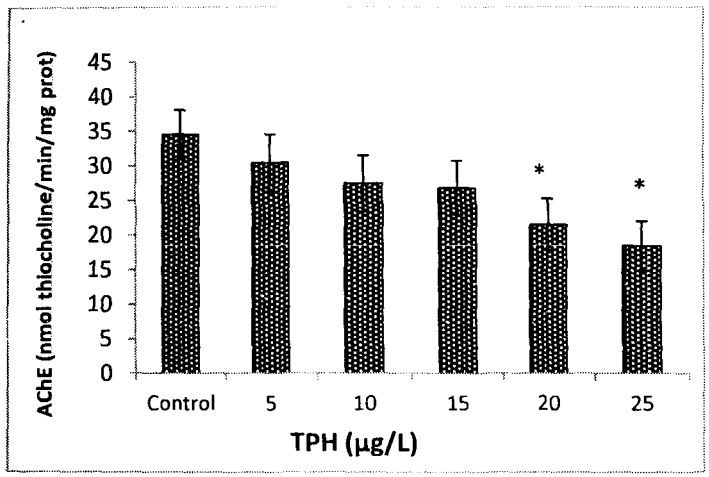
Fig. 4.2. Malondialdehyde (MDA) in the soft tissues of *M. casta* (Mean ± SD) exposed to various concentration of **(E)** cadmium chloride **(F)** lead acetate and **(G)** pollutant mixture



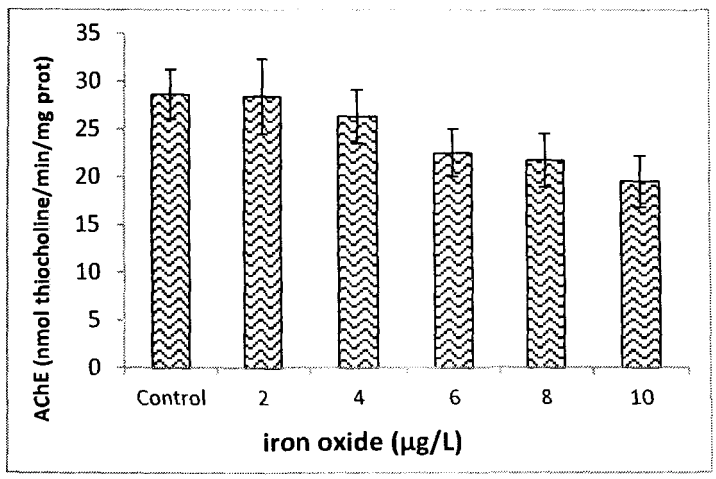
Note: * $p < 0.05$

Fig. 4.3. Acetylcholinesterase (AChE) activity in the soft tissues of *M. casta* (Mean ± SD) exposed to various concentrations of **(A)** TPH and **(B)** iron oxide

A



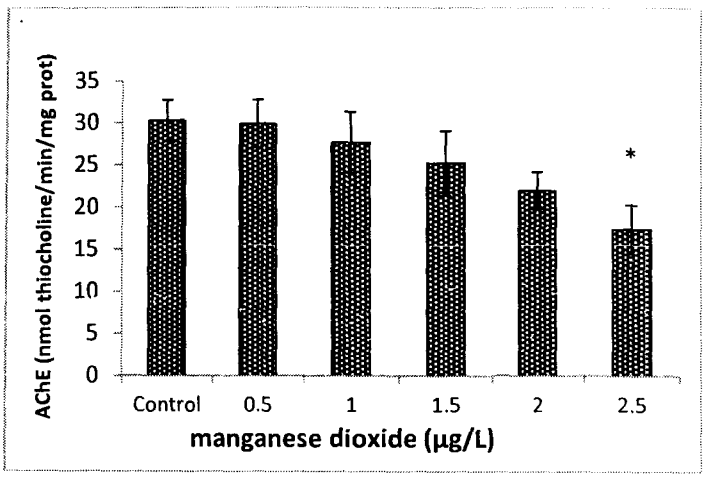
B



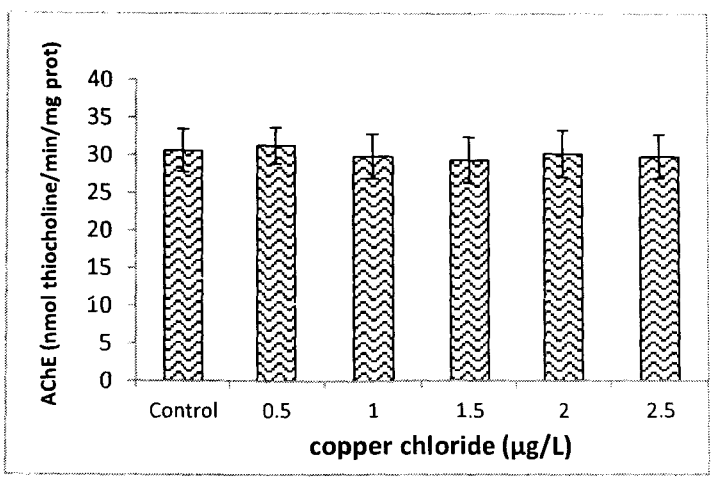
Note: * $p < 0.05$

Fig. 4.3. Acetylcholinesterase (AChE) activity in the soft tissues of *M. casta* (Mean ± SD) exposed to various concentrations of **(C)** manganese dioxide and **(D)** copper chloride

C

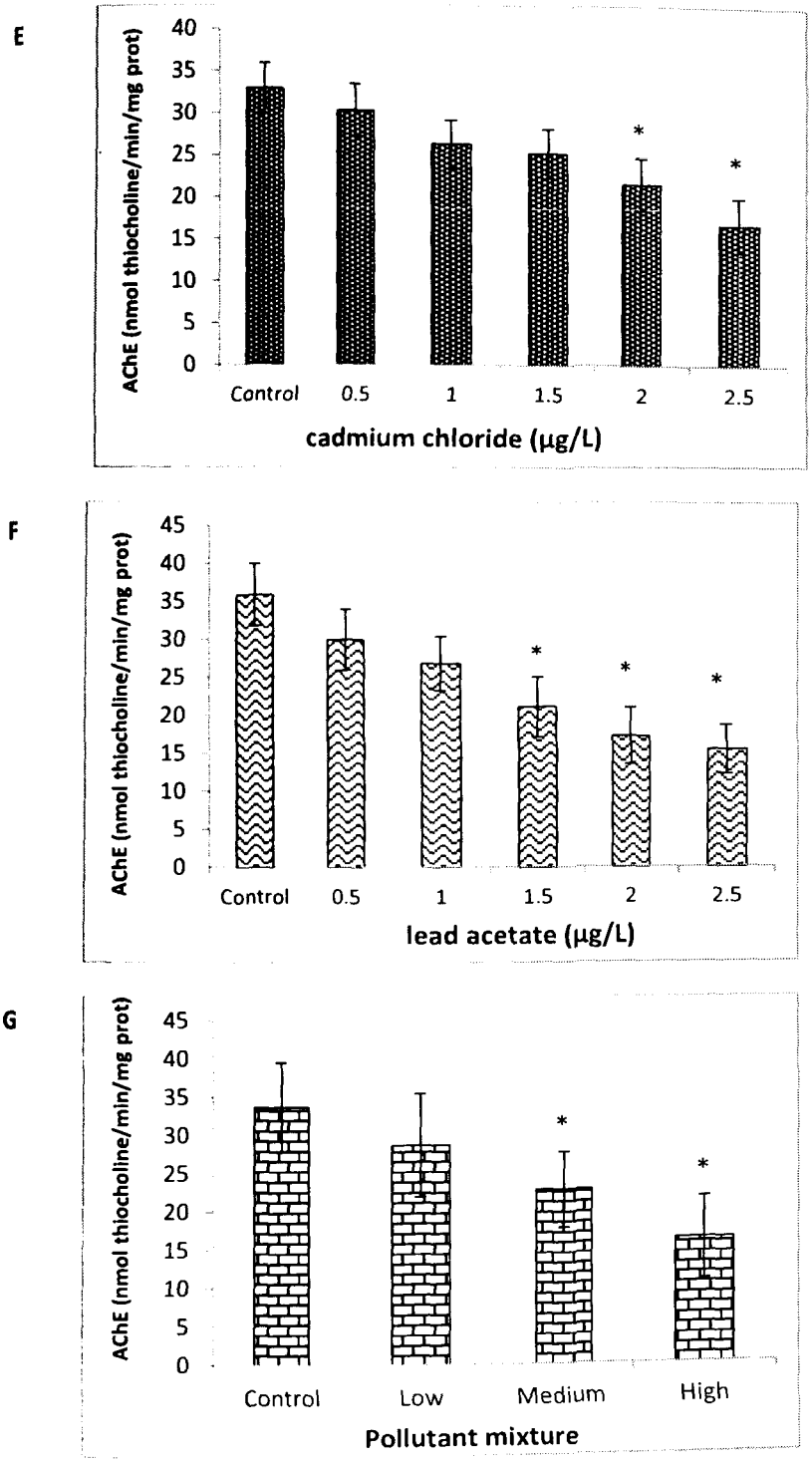


D



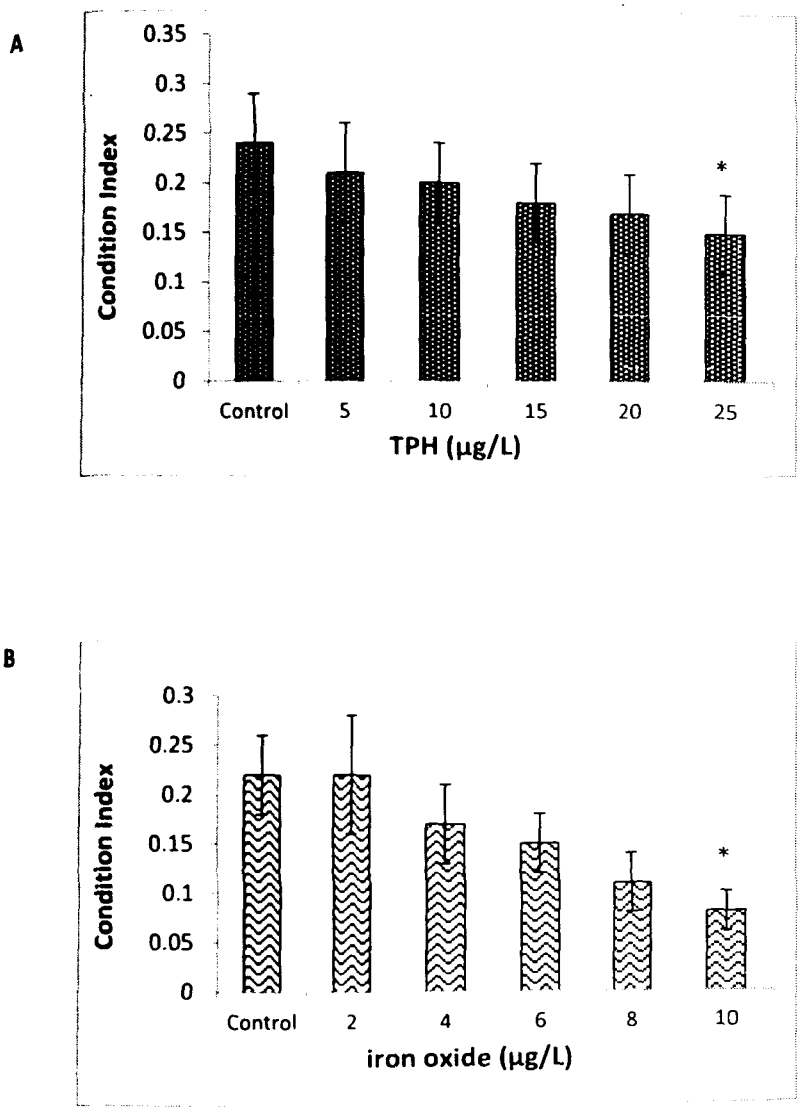
Note: * $p < 0.05$

Fig. 4.3. Acetylcholinesterase (AChE) activity in the soft tissues of *M. casta* (Mean ± SD) exposed to various concentrations of **(E)** cadmium chloride **(F)** lead acetate and **(G)** pollutant mixture



Note: * $p < 0.05$

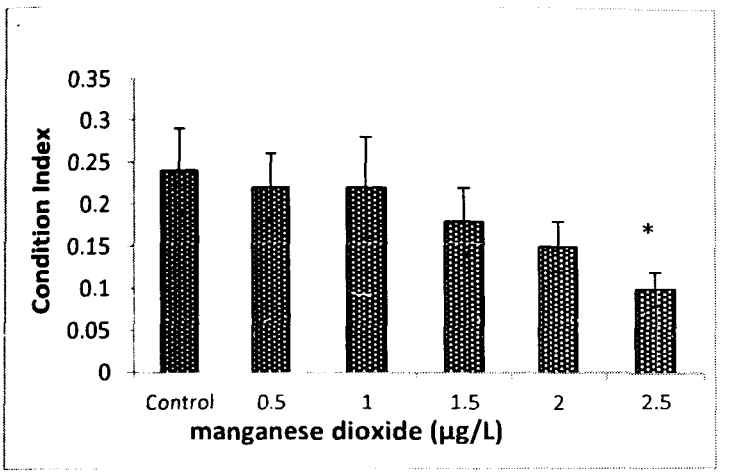
Fig. 4.4. Condition index (CI) of *M. casta* (Mean ± SD) exposed to various concentrations of **(A)** TPH and **(B)** iron oxide



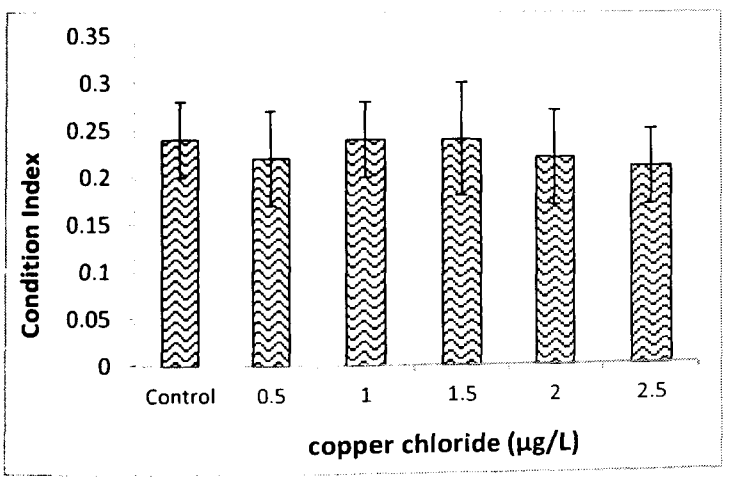
Note: * $p < 0.05$

Fig. 4.4. Condition index (CI) of *M. casta* (Mean ± SD) exposed to various concentrations of **(C)** manganese dioxide and **(D)** copper chloride

C

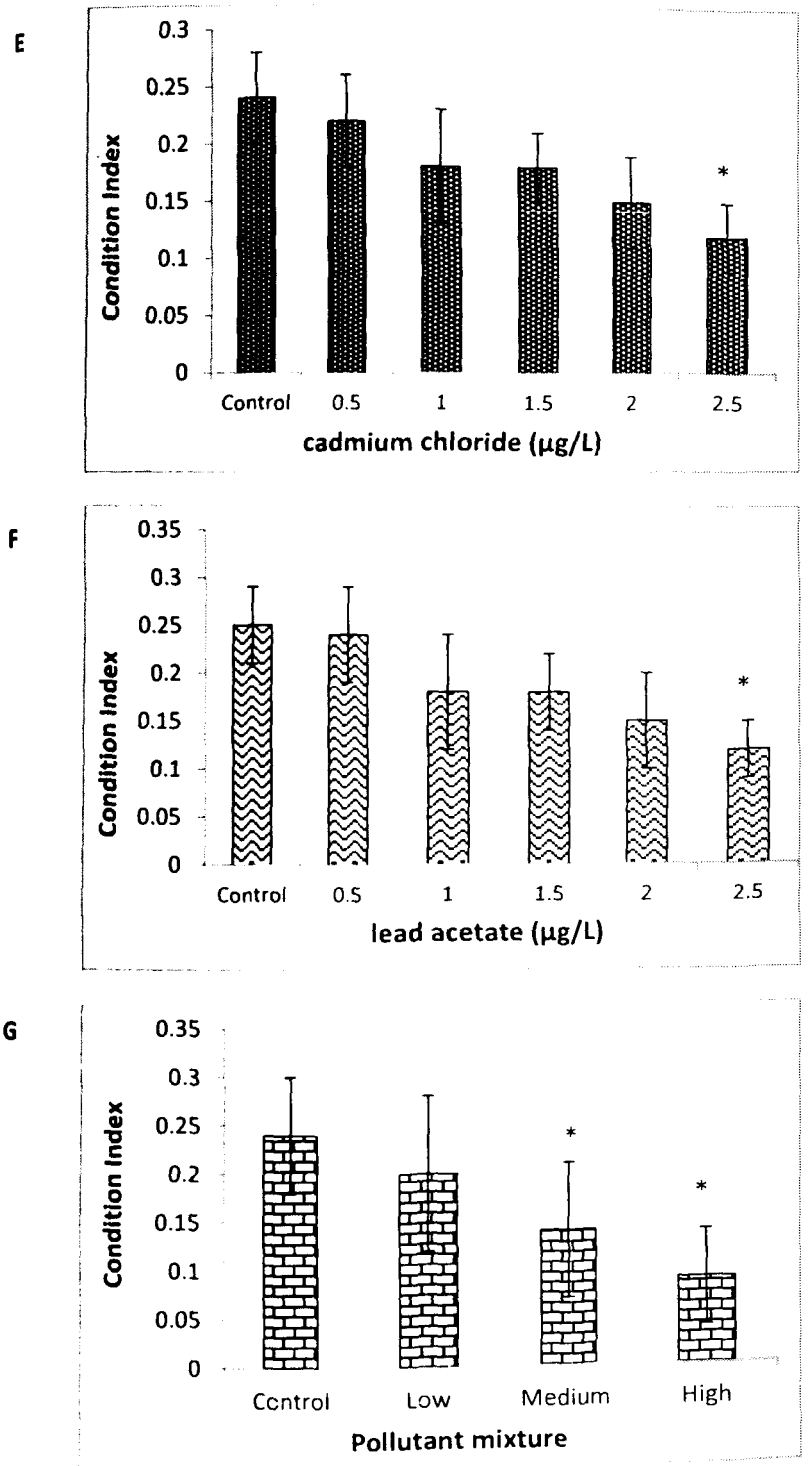


D



Note: * $p < 0.05$

Fig. 4.4. Condition index (CI) of *M. casta* (Mean ± SD) exposed to various concentrations of **(E)** **cadmium chloride (F) lead acetate and (G)** pollutant mixture



Note: * $p < 0.05$

Table 4.1. Correlation matrix between the associations of the biomarker responses in *M. casta* exposed to **(A)** TPHs and **(B)** iron oxide

A	% MNi	% Tail DNA	CAT	AChE	MDA	CI
% MNi	-					
%Tail DNA	0.93***	-				
CAT	0.90***	0.92***	-			
AChE	-0.76**	-0.80**	-0.72**	-		
MDA	0.85***	0.89***	0.89***	-0.85**	-	
CI	-0.78**	-0.86***	-0.82**	0.79**	-0.76**	-

B	% MNi	% Tail DNA	CAT	AChE	MDA	CI
% MNi	-					
%Tail DNA	0.85***	-				
CAT	0.75**	0.76**	-			
AChE	-0.05	-0.19	-0.19	-		
MDA	0.69*	0.65*	0.59*	-0.25	-	
CI	-0.78**	-0.76**	-0.52	0.17	-0.36	-

Note: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

Table 4.1. Correlation matrix between the associations of the biomarker responses in *M. casta* exposed to **(C)** manganese dioxide and **(D)** copper chloride

C	% MNi	% Tail DNA	CAT	AChE	MDA	CI
% MNi	-					
%Tail DNA	0.89***	-				
CAT	0.56*	0.59*	-			
AChE	-0.75**	-0.78**	-0.25	-		
MDA	0.78**	0.85***	0.55*	-0.76**	-	
CI	-0.81**	-0.86***	-0.50	0.69**	-0.75**	-

D	% MNi	% Tail DNA	CAT	AChE	MDA	CI
% MNi	-					
%Tail DNA	0.21	-				
CAT	0.07	0.20	-			
AChE	0.12	0.08	-0.09	-		
MDA	-0.15	0.14	-0.16	-0.14	-	
CI	-0.10	-0.09	-0.08	0.08	0.14	-

Note: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

Table 4.1. Correlation matrix between the associations of the biomarker responses in *M. casta* exposed to (E) cadmium chloride and (F) lead acetate

E	% MNi	% Tail DNA	CAT	AChE	MDA	CI
% MNi	-					
%Tail DNA	0.95***	-				
CAT	0.92***	0.92***	-			
AChE	-0.93***	-0.94***	-0.92***	-		
MDA	0.95***	0.96***	0.89***	-0.92***	-	
CI	-0.86***	-0.90***	-0.87***	0.87***	-0.85***	-

F	% MNi	% Tail DNA	CAT	AChE	MDA	CI
% MNi	-					
%Tail DNA	0.89***	-				
CAT	0.90***	0.91***	-			
AChE	-0.86***	-0.92***	-0.87***	-		
MDA	0.91***	0.84**	0.92***	-0.82**	-	
CI	-0.85***	-0.90***	-0.86***	0.85***	-0.87***	-

Note: ** $p < 0.01$; *** $p < 0.001$

Table 4.1. Correlation matrix between the associations of the biomarker responses in *M. casta* exposed to **(G)** the pollutant mixture

G	% MNi	% Tail DNA	CAT	AChE	MDA	CI
% MNi	-					
%Tail DNA	0.95***	-				
CAT	0.92***	0.90***	-			
AChE	-0.94***	-0.90***	-0.91***	-		
MDA	0.95***	0.94***	0.95***	-0.85***	-	
CI	-0.87***	-0.89***	-0.90***	0.87***	-0.86***	-

Note: *** $p < 0.001$

4.5. Discussion

The bivalves exposed to increasing concentrations of TPHs in the present study exhibited significant changes in the activities of CAT, MDA, AChE as well as condition index at the highest concentrations of the pollutants. Our results are on par with that of Frouin *et al.* (2007) in which significant increases of the activities of both CAT and MDA were observed in clams (*Mya arenaria*) exposed to PAHs for a period of 30 days. CAT activity was also found to be elevated in the tissues of the blue mussel (*Mytilus edulis*) exposed to the PAHs benzo[a]pyrene and fluoranthene (Eertman *et al.*, 1995). Our results also find similarity with that of Giannapas *et al.* (2012) in which MDA was found to be significantly increased in the tissues of the mussels *Mytilus galloprovincialis* exposed to PAHs such as phenanthrene and anthracene as well as a combination of both PAHs.

In the present study, Fe and Cu did not induce significant oxidative stress in the bivalves. Zhang *et al.* (2010) also reported non-significant oxidative stress measured by catalase assay in bivalves (*Chlamys farreri*) exposed to environmentally relevant concentrations of Cu. AChE activity was also not found to be significant in the Fe-exposed bivalves. Kádár *et al.* (2010) also reported non-significant AChE activity in the gill cells of the blue mussel *Mytilus* sp. exposed to Fe compared to the negative control.

Besides inducing indirect DNA damage via ROS and inhibiting DNA repair processes, Cd also interferes with enzymes of the cellular antioxidant system (Waisberg *et al.*, 2003). CAT activity in *M. casta* was found to be significantly increased at the 2 and 2.5 µg/L concentrations of Cd. Similar observations were also recorded by Boudjema *et al.* (2014) in which CAT activity was found to be elevated in the soft tissues of brown mussels (*Perna perna*) exposed to Cd. In this study, significant levels of MDA were also measured in the soft

tissues of bivalves exposed to Cd. These observations are similar to the findings of Geret *et al.* (2002) in which MDA production was found to be elevated in the gills of the clam *Ruditapes decussatus* experimentally exposed to Cd. The activity of AChE in the clams in the present study also showed a decreasing trend with increasing concentration of Cd. Cunha *et al.* (2007) also observed a decrease in the activity of AChE in the soft tissues of two marine gastropods (*Monodonta lineata* and *Nucella lapillus*) exposed to Cd. Cd is thus a potent toxicant that is able to interfere with antioxidant defenses and can cause neurotoxicity to aquatic organisms at low concentrations.

In the present study, Pb was able to induce significant oxidative stress in the soft tissues of the bivalves. These findings are similar to that of Boudjema *et al.* (2014) in which significant elevation of CAT was reported in the brown mussel (*Perna perna*) experimentally exposed to Pb. Our results are also on par with that of Zhang *et al.* (2010) in which bivalves (*Chlamys farreri*) exposed to environmentally relevant concentrations of Pb exhibited significant lipid peroxidation in their soft tissues. Lead is known to contribute to oxidative stress via the formation of ROS. In case of Pb toxicity, these ROS cause lipid peroxidation, measured by the levels of MDA and may lead to cell membrane damage (Yiin and Lin, 1995; Ercal *et al.*, 2001). The activity of AChE was also found to decrease in a concentration-dependent manner in the present study. Labrot *et al.* (1995) also observed a decrease in the AChE activity in a mollusc (*Corbicula* sp.) exposed to Pb under laboratory conditions.

Our results find similarity with that of Wang *et al.* (2011) in which they observed an increase of oxidative stress enzymes in bivalves (*Ruditapes phillipinarium*) exposed to a mixture of Cd and benzo[a]pyrene for a period of 21 days.

A significant negative correlation was observed between the MN test and CI (R = -0.86) as well as between the comet assay and CI (R = 0.9). A similar negative correlation between condition index and tissue levels of environmental contaminants of *Littorina littorea, Mytilus edulis* and *Cerastoderma edule* in a river system (Milford Haven Waterway) of Wales, UK was reported by Langston *et al.* (2012). This decrease of condition of the organism may be attributed to altered DNA function and thereby resulting in an altered protein function which is ultimately required for normal physiological processes. The physiological state of the bivalve can also lead to changes in its feeding activity thereby altering its life cycle as a consequence (Babarro *et al.*, 2000). Another reason for the decrease in the CI ratio could possibly be the survival adaptive response of *M. casta* wherein they reduce the filtration rate or closure of the shell on exposure to contaminants (Akcha, 2000).

4.6. Conclusions

Based on the results obtained in this study, the biomarkers of oxidative stress such as catalase and malondialdehyde activity can be reliably used to assess early signs of toxicity by environmental contaminants. Further, biomarkers such as acetylcholinesterase activity can be used to assess the neurotoxicity of environmental contaminants such as Cd, Pb and TPHs. The condition index can be used as a simple and effective biomarker to assess the physiological condition of the organisms collected from sites of anthropogenic stress. Pollution indicators and genotoxicity tests, combined with other physiological or biochemical parameters can therefore be used for efficient monitoring of aquatic ecosystems in Goa.

Summary

The present study reports the pollution status of the Goan Coast by quantifying the levels of total petroleum hydrocarbons (TPHs) and trace metals in the water as well as the sediment of probable polluted sites (Vasco, Dona Paula and Miramar) and comparing these values with a control / pristine unpolluted site (Palolem). The probable polluted sites are influenced by considerable anthropogenic inputs such as mining activities, including transport of ores and release of industrial effluents. Water and sediment samples were collected from these sites twice per season (Pre-monsoon, Monsoon and Post-monsoon) from June 2012 to May 2014. The quantity of total PAHs and heavy metals present in the water samples and tissues were analysed using spectrofluorometry and atomic absorption spectrophotometry (AAS), respectively. Physico-chemical parameters of water such as temperature, pH, salinity, dissolved oxygen, phosphates and nitrates were also recorded to supplement the data on trace metals and TPHs for statistical analyses. The concentration of trace metals and TPHs in the water and sediment were found to be highest at Vasco followed by Dona Paula and the least in Miramar.

Genotoxicity studies were carried out in the natural populations of estuarine fauna inhabiting these waters. In this study, finfish viz. *Arius arius* (Thread-fin sea catfish) and *Johnius dussumieiri* (Sin croaker) as well as the shell fish *Meretrix casta* and *Meretrix ovum* (Estuarine clams) were collected from each of these sites and the extent of DNA damage was evaluated in them employing the micronucleus test and alkaline single cell gel electrophoresis (comet) assay. The genotoxicity tests were supplemented by the quantification of TPHs and heavy metals accumulated in various tissues these organisms. The results showed significant levels of DNA damage as micronuclei (MN) and % tail DNA in the fishes collected from Vasco, Dona Paula and Miramar. Significant concentrations of

TPHs and heavy metals such as iron (Fe) and manganese (Mn) were also observed in the tissues of the fishes collected from these sites. A highly positive correlation was observed between the pollutant levels and DNA damage in all the specimens collected from the polluted sites suggesting the genotoxic nature of these pollutants.

In order to understand the extent of DNA damage induced by trace metals and TPHs, *Meretrix casta* were exposed to these pollutants under laboratory conditions. In this study, bivalves were collected from the intertidal zone with the help of skilled local fishermen from Palolem. They were transported alive to the laboratory and were then allowed to acclimatize in ordinary seawater from Palolem for 30 days. Bivalves were distributed in groups, each containing 10 individuals and were exposed to various concentrations of Fe, Mn, Cu, Cd, Pb and TPHs for 15 days. DNA damage in the form of micronuclei and % tail DNA was observed in *M. casta* exposed to Fe, Mn, Cd, Pb and TPHs. Cu did not induce any significant DNA damage. Further, the mixture of all these pollutants also induced significant DNA damage compared to the controls. A high positive correlation was observed between MN and % tail DNA which measure double and single stranded breaks of DNA respectively.

In order to assess the possible role of the pollutants on various biochemical and physiological parameters which induce DNA damage, bivalves were exposed to various concentrations of heavy metals and TPHs in the laboratory and their catalase (CAT), malondialdehyde (MDA), acetylcholinesterase (AChE) activity and condition index (CI) were measured. *M. casta* were distributed in groups, each containing 10 individuals and were exposed to different concentrations of Fe, Mn, Cu, Cd, Pb and TPHs for 15 days. Catalase assay and Malondialdehyde assay were performed to assess the oxidative stress induced in the bivalves. Acetylcholinesterase activity was tested to evaluate the neurotoxic potential of the pollutants.

The condition index was performed to measure the physiological condition of exposed bivalves. Fe did not induce significant changes in CAT, MDA and AChE, but a significant decrease in the condition index was observed at the highest dose. Mn was found to significantly decrease the AChe activity at the highest dose. Subsequently an increase of MDA was observed at the highest dose of Mn. Cu did not induce significant changes in any of the parameters. However, Cd, Pb and TPH and the mixture of all the pollutants caused significant increases in CAT and MDA activity, decrease in AChe activity and a decrease in the condition index of the exposed bivalves.

In conclusion, the data for genotoxicity recorded in these finfishes and shellfishes can be used as reference values for future genotoxicity, biochemical or physiological studies of these study sites. Monitoring of these sites is therefore essential to know the "health" of the aquatic environment. Pollution indicators and genotoxicity tests, supplemented with data from oxidative stress, biochemical or physiological parameters can be used for efficient monitoring of aquatic ecosystems in Goa.

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Publications

PUBLICATIONS

Research articles

- D'costa **AH**, Shyama SK, Praveen Kumar MK. 2017. Bioaccumulation of trace metals and total petroleum and genotoxicity responses in a wild fish population as indicators of marine pollution. Ecotoxicology and Environmental Safety. 142: 22-28 (Impact factor 3.974)

- D'costa **AH**, Shyama SK, Praveen Kumar MK, Furtado S. 2017. The Backwater Clam (*Meretrix casta*) as a bioindicator species for monitoring the pollution of an estuarine environment by genotoxic agents. Mutation Research/Genetic Toxicology and Environmental Mutagenesis. 825: 8-14 (Impact factor 1.996)

- D'costa **AH**, Shyama SK, Praveen Kumar MK, Furtado S. 2017. Genotoxic and Biochemical biomarker responses in *Meretrix casta* exposed to environmentally relevant concentrations of Cadmium. Biosensors, Biomarkers & Diagnostics. 2(1): 1-7 (Impact factor NA)

Book chapters

- Praveen Kumar MK, **D'costa A**, Shyama SK. 2016. Genotoxic Biomarkers as Indicators of Marine Pollution. *In*: Marine Pollution and Microbial Remediation, editors Naik MM, Dubey SK. Springer Nature. 17: pp 263-270

Papers / Posters presented in conferences

- **D'costa AH** and Shyama SK. Monitoring the pollution status of Goan Coast using genotoxicity studies in the fish, *Arius arius*. Awarded 1st prize at the conference on "Ecosystem: Emerging Issues", held at Poornaprajna College, Udupi in association with Karnataka Science and Technology Academy (KSTA). 09-10 January 2015

- **D'costa AH** and Shyama SK. Evaluation of the pollution status of the Goan Coast using genotoxicity biomarkers in the fish, *Johnius dussumieri*, at the national seminar on "Life and Life Processes: Sustainable Development", held at the Department of Zoology, Goa University, 19-21 February 2015

- **D'costa A**, Shyama SK, Praveen Kumar MK. Evaluation of the pollution status of the Goan coast using genotoxicity biomarkers in the bivalve, *Meretrix casta*, at the International conference on "Advances in Cellular, genomic and Epigenomic Insights on Environmental Mutagenesis and Health" organized by Environmental Mutagen Society of India (EMSI) and School of Life Sciences, Manipal University, Manipal, 27-29 January 2017

- **D'costa A**, Shyama SK, Braganza K. Evaluation of the genotoxic effects of environmentally relevant concentrations of cadmium in estuarine backwater clam *Paphia malabarica*, at the seminar on "New Perspectives in Biosciences" organized by Department of Microbiology, Goa University, 07 December 2017

• **D'Costa A**, Shyama SK, Praveen Kumar MK. Genotoxicity evaluation of environmentally relevant concentrations of total petroleum hydrocarbons in the estuarine backwater clam *Meretrix casta*, at the "International Conference on Advancement in Science & Technology (ICAST 2018)" organized by Indian Japan Society for the Promotion of Science (JSPS) and Department of Physics, Visva-Bharati University, Santiniketan, West Bengal, 03-04 September 2018

Winzinger et al., 2007). The negative correlation may depend on the fish species chosen and / or on the nature of the assays.

Micronuclei result from acentric chromosome fragments or whole chromosomes lagging behind during metaphase/anaphase transition induced by clastogens or by spindle dysfunctions, respectively. The single-cell gel electrophoresis (SCGE) is a rapid and sensitive technique that detects DNA strand breaks, measuring the migration of nicked DNA fragments from immobilized individual cell nuclei. Both these tests are routinely used as biomarkers for monitoring aquatic pollution by genotoxic contaminants and can be combined with other physiological and biochemical biomarkers to fully assess the pollution status of various water bodies (Praveen Kumar et al., 2015; Bolognesi and Cirillo, 2014).

We hypothesized that pollutants such as trace metals and petroleum hydrocarbons induce genotoxicity in *Arius arius* and that DNA damage could be affected by seasonality. Thus, the primary goal of this study was to report the genotoxicity status of this native fish population at a probable polluted site (Vasco) and an unpolluted site (Palolem) and also to estimate the quantities of total petroleum hydrocarbons (TPHs) and trace metals such as Fe, Mn, Cu, Cd and Pb in the water and sediment as well as the tissues of the fish. The rationale for selecting these pollutants is that they are found in the waters as a result of mining from iron and manganese mine sites and effluents released from agrochemical industries and induce toxicity and biochemical changes in estuarine and marine fauna. We further assessed the relationship between DNA damage and pollutants from the tissues of fish at different sites during various sampling seasons.

2. Materials and methods

2.1. Study sites

Palolem and Vasco were selected as the unpolluted (reference) and polluted sites, respectively, along the coast of the Indian state of Goa (Fig. 1), based on the reports of Sarkar et al., (2008, 2010) and Sarker and Sarkar (2015). Palolem is a clean, pristine beach with no known industrial activity at this site. Vasco, an industrial hub, is dependent on the Mormugao harbour for most of its economic activities. This harbour serves as a port for carrier barges, ore barges, oil liners and cruise liners. Thus, the water and sediment around this area suffers considerable anthropogenic stress.

2.2. Sample collection

2.2.1. Water and sediment collection

Surface and bottom water (2 L each) were collected with the help of a Niskin Sampler. The temperature, pH and salinity of the water samples were immediately recorded with the help of an automated water and sediment analysis kit (Labtronics, India) consisting of probes for measuring the required parameter. Water samples for dissolved oxygen were taken in amber-coloured bottles (300 ml) and fixed with Winkler's solution. The samples for the analysis of phosphates, nitrates, trace metals and TPHs were stored in clean, labelled polypropylene bottles. Sediment samples (~ 500 g) were collected with the help of a Van-Veen Grab and stored in clean, labelled polythene bags. These samples were stored at 4 °C and carried to the laboratory for further analysis. Samples were collected from three sampling stations in triplicates at each site during the pre-monsoon, monsoon and post-monsoon periods from March 2012 to February 2014.

2.2.2. Fish collection

Arius arius (Thread-fin sea catfish) was chosen for the present study due to its bottom feeding niche, availability in Goan waters all year round and its consumption by a majority of people living along the coast. Both sexes of fish were collected with the help of a trawl net (mesh size 40 mm), which was mechanically operated by a trawler at the sites as described for water and sediment collection. The sample size of the fish collected from these sites and across the seasons is given in Table 1. Blood was immediately drawn from the caudal vein, smeared on clean glass slides and stored in slide boxes. Excess blood was stored in microfuge tubes containing phosphate buffered saline (pH 7.4) at 4 °C. The fish were then stored in clean labelled polythene bags and transported in ice bags to the laboratory for further analyses of the metal and TPH content in their tissues.

2.3. Analysis of samples

2.3.1. Physico-chemical parameters

The physico-chemical parameters temperature, pH, salinity and dissolved oxygen were analysed and recorded as mentioned earlier. Phosphates were analysed Nitrates were analysed spectrophotometrically using the Brucine method (EPA, 1971). Phosphates were estimated by adding ammonium molybdate and stannous chloride to the water sample and reading the intensity of a blue coloured complex at 690 nm (Bureau of Indian Standards, 1988).

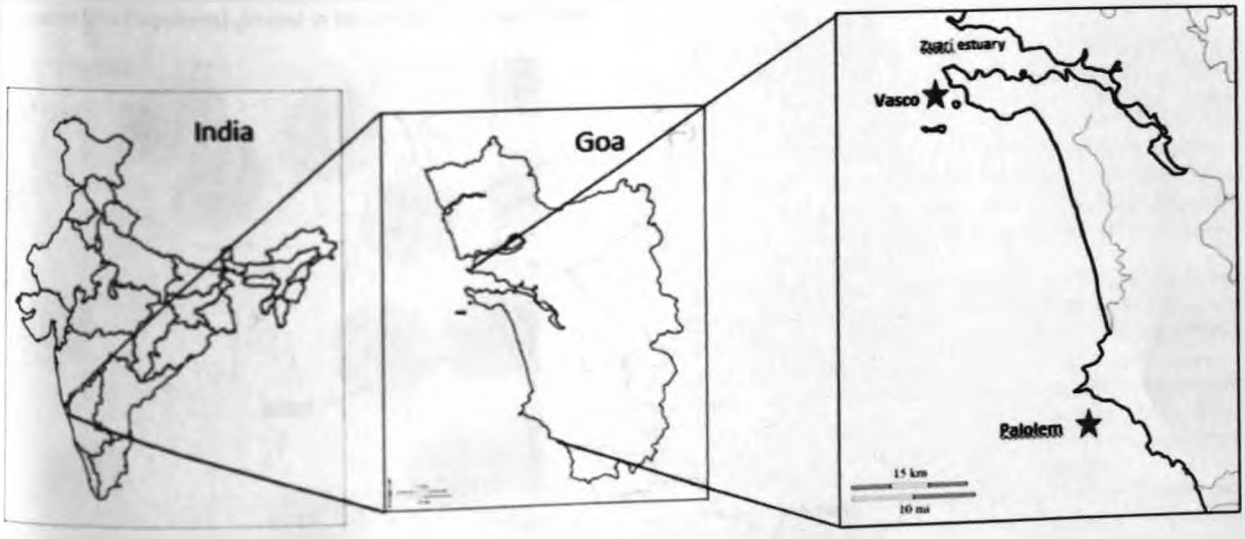

Fig. 1. Sampling sites at Vasco and Palolem along the coast of Goa, India.

Table 1
Total number of *A. aruana* collected from the study sites in different seasons along with the coordinates of the sampling stations.

	Palolem			Vasco		
	Station 1 15°00'17" N, 74°00'34" E	Station 2 15°00'08" N, 74°01'16" E	Station 3 14°59'53" N, 74°01'22" E	Station 1 15°25'40" N, 73°47'17" E	Station 2 15°24'57" N, 73°47'27" E	Station 3 15°24'02" N, 73°46'38" E
Pre-Monsoon (2012)	41	32	38	29	36	47
Monsoon (2012)	24	27	22	25	23	27
Post-Monsoon (2012–13)	37	30	25	27	31	26
Pre-Monsoon (2013)	29	22	35	33	24	29
Monsoon (2013)	37	28	26	23	40	34
Post-Monsoon (2013–14)	30	29	39	32	26	28

2.3.2. Micronucleus test

The micronucleus test was carried out as detailed by Baršiene et al., (2004, 2006). A drop of blood from the caudal vein was directly smeared on glass slides and air-dried. The smears were fixed in methanol for 10 min, stained with 5% Giemsa for 15 mins and allowed to dry. The frequency of micronuclei (MNi) was recorded by scoring 2000 cells per fish at 1000 × magnification, using an Olympus BX53 microscope. MNi were identified according to the following criteria: (1) spherical or ovoid-shaped extra nuclear bodies in the cytoplasm (2) a diameter of 1/3–1/20 of the main nucleus (3) non-refractory bodies (4) colour texture and optical features resembling those of the nucleus, and (5) the bodies completely separated from the main nucleus (Fig. 2).

2.3.3. Single cell gel electrophoresis (comet assay)

The comet assay was carried out using the procedure outlined by Lee and Steinert (2003). 20 µl of blood, diluted to approximately 10,000 cells with phosphate buffer saline (pH 7.4) was embedded in low melting agarose (LMA) on frosted microscopic slides and then placed in a cold lysing solution (2.5 M NaCl, 100 mM Na_2EDTA, 10Mm Tris, 10% DMSO And 1% Triton-X pH 10) at 4 °C, overnight. All the steps were done in dim light to prevent photo-oxidation of DNA. The slides were then placed in electrophoresis buffer (pH 10) for 15–20 min to allow the DNA to unwind. Electrophoresis was then performed under alkaline conditions (pH 10) for 30 min at 300 mA, 25 V. The slides were placed in neutralization buffer (400 mM Tris base, pH 7.5) for 5 min. The gel containing DNA was stained with ethidium bromide and examined using a fluorescence microscope with a green filter at 200x magnification. Randomly selected non-overlapping cells were screened and analysed with the help of computer software, CASP (Konca et al., 2003) and the % tail DNA was recorded (Fig. 3A and B). The % tail DNA is the amount of DNA (in percent) present in the tail of the "comet" and is used as a measure of DNA damage (Kumaravel and Jha, 2006).

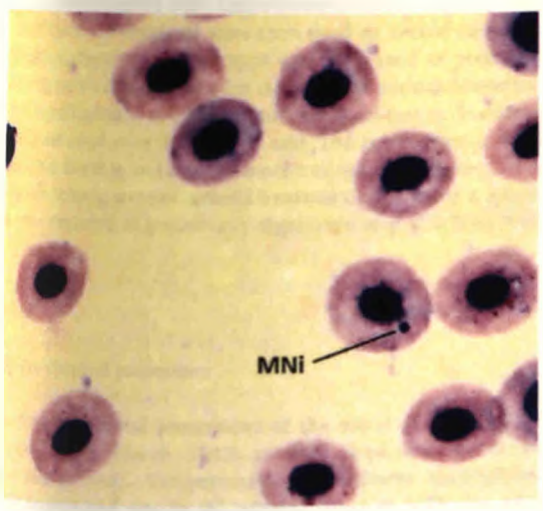

Fig. 2. Erythrocyte of *A. aruana* with micronucleus.

2.4. Sample preparation for TPH and trace metal analysis

Muscle, gill and liver tissues were collected from the fish and were analysed for TPH and trace metals.

2.4.1. Total petroleum hydrocarbon (TPH) analysis

Tissue samples: Tissues were homogenized with hexane in a Potter-Elvehjem homogenizer. The samples were dried by passing through a column of anhydrous sodium sulphate and made to a final volume of 25 ml with hexane. The concentration of total petroleum hydrocarbon was analysed spectrofluorometrically (Shimadzu) with excitation at 310 nm and emission at 360 nm. The values were expressed as µg/g of wet tissue (Ansari et al., 2012).

Water samples: 2 ml of hexane was added to 20 ml of water and shaken for 20 min in capped tubes. This mixture was allowed to stand to allow the two phases to separate. The hexane layer was obtained and dried under a stream of nitrogen gas. The sample was then reconstituted with 1.5 ml of HPLC-grade ethanol and 1.5 ml of de-ionized water and stored at 4 °C in dim light (Ramachandran et al., 2006). The TPH concentrations were analysed spectrofluorometrically as mentioned for tissue samples.

Sediment samples: 10g of sediment was mixed with tetrohydrofuran, shaken vigorously and placed in an ultrasonicator bath for 1–2 h. The samples were then centrifuged at 2000 rpm for 30 min and the supernatant was evaporated using a stream of nitrogen gas. The residue was then re-dissolved in 20 ml of hexane and was analysed spectrofluorometrically (Telli-Karakoç et al., 2002).

2.4.2. Trace metal analysis

Tissue samples: Concentrations of iron (Fe), manganese (Mn), copper (Cu), cadmium (Cd) and lead (Pb) were determined by Flame Atomic Absorption Spectrophotometry (Shimadzu) according to Begum et al. (2013). Briefly, tissue samples were digested with 10 ml of 14 M nitric acid, 5 ml of 13 M perchloric acid and 5 ml of deionized water in teflon tubes and heated at 150 °C for 2 h. The solution was then allowed to evaporate to 5 ml in a Teflon beaker using a hot plate. The solution was cooled and was diluted upto 50 ml using deionized water. Results were expressed as µg/g of wet tissue.

Water samples: Water samples acidified with HNO_3 were mixed with 1% ammonium pyrolidine dithiocarbamate (APDC) and methyl isobutyl ketone (MIBK) in a separating funnel and shaken for 5 min. This mixture was allowed to stand for about 15–20 min to allow phase separation. The organic layer obtained was transferred to another separating funnel and back-extracted with 4N HNO_3. The lower aqueous phase was collected in a centrifuge tube (Satyanarayan et al., 2007). Results were expressed as µg/L of sample.

Sediment samples: 1–2 g of dried sediment were digested with aqua regia (1:3 concentrated HNO_3 and HCl) and heated on a hot plate for 2 h. The digestate was then filtered and diluted to a final volume of

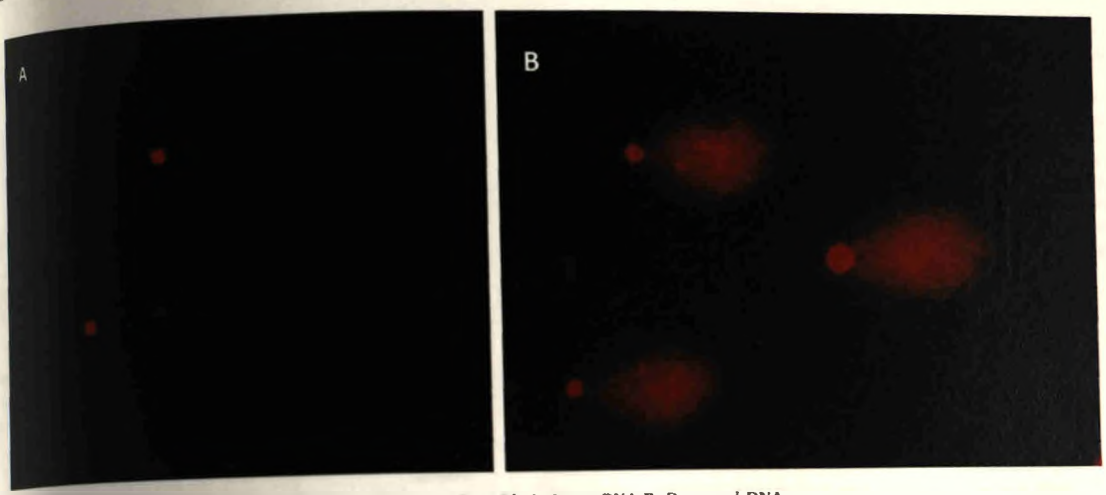

Fig. 3. Erythrocytes of *A. arius* with **A.** Intact DNA **B.** Damaged DNA.

l/ml with distilled water (USEPA, 1996). Results were expressed as g of dried sample.

Quality assurance and quality control

The appropriate quality assurance methods of sample preparation, ndling and preservation were carried out in accordance with US EPA rocedures. The certified reference material for TPH estimation was mbay High crude oil and was used to prepare the calibration curve. 'raceCERT certified reference metals for AAS (Sigma)" were used for ce metal estimation.

Statistical analyses

Statistical analysis was performed using IBM© SPSS 23 statistical ta are package. The relationship between MNi and DNA damage was alysed employing Pearson's correlation analysis. The data were tested meet the assumptions of normality using Shapiro-Wilk's test and mogeneity using Levene's test and were subsequently arcsine transmed to improve linearity. These values were then used for various rametric tests. To reduce the number of variables that may be interrelated, a principle components analysis (PCA) was performed using ificant concentrations in the environment, pollutant concentrations the different tissues of *A. arius* and physico-chemical parameters. The rious seasons and sites were converted to dummy variables and also ed in the PCA. In order to enhance the interpretation of factor ads, Varimax rotation with Kaiser Normalization was used and the ctor coefficients so obtained were then used to derive factor scores. ctors with eigenvalues greater than one were used as predictors for ltiple regression analysis in order to eliminate multicollinearity. The tistical significance of the data of micronucleus test and comet assay re analysed employing Student's t- test. The correlation coefficients tween DNA damage and tissue concentrations of trace metals across e sites and seasons were compared between tissues using a z-test. The sults were regarded as statistically significant at $p < 0.001$, 0.01 and 5.

Results

Physico-chemical parameters

The physico-chemical parameters of the water collected from the dy sites during March 2012 to Feb 2014 are given in A1 pplementary Data). Temperature was uniform throughout the mpling seasons and was in the normal range at both the sites. pH ues of Palolem water samples were also in the normal range, but that of Vasco was slightly lower during the pre-monsoon seasons. Salinity ranged from 31 to 35 ppt throughout the sampling seasons at both the sites. Dissolved oxygen concentrations at Palolem were significantly higher during the monsoon seasons compared to that of Vasco in all the sampling seasons. Phosphate concentrations were found to be non-significant at both Palolem and Vasco. However, nitrate concentrations were found to be significantly higher at Vasco compared to Palolem.

3.2. TPHs and trace metals

The concentrations of total petroleum hydrocarbons and trace metals viz Fe, Mn, Cu, Cd and Pb in the water as well as sediment from Palolem and Vasco are presented in A2-7 (Supplementary Data). Water from Vasco showed higher concentration of trace metals than that of Palolem. Fe and Mn were significantly high in the sediment samples from Vasco compared to Palolem in all the seasons. The value of Cu was also high in the sediment from Vasco in the pre-monsoon (2012) season. Cadmium concentrations were also found to be high in the water, and to a larger extent, in the sediment samples from Vasco.

The concentration of TPHs and trace metals in the various tissues (muscle, gill and liver) of *A. arius* are presented in A9. The highest concentration of TPHs was found in the livers of fish collected from Vasco. The values of Fe were significantly high ($p = 0.043$, $p = 0.008$) in the tissues of the fish collected from Vasco. Mn concentrations were observed to be significantly high ($p = 0.032$) in gill and liver tissues from Vasco compared to Palolem in all the seasons. The concentration of Cu was significantly high ($p = 0.041$) in the tissues of *A. arius* in the monsoon season of 2012. Cd was also found to be significantly high ($p = 0.044$) in tissues of *A. arius* collected from Vasco compared to Palolem. The concentration of Pb was also significantly high in the tissues of the fish, particularly in the gills of *A. arius* in all the seasons compared to Palolem.

3.3. Micronucleus test

Fig. 4 represents the incidence of micronuclei (MNi) in the erythrocytes of *A. arius*. Significantly high values ($p < 0.024$) of MNi were noted in the erythrocytes of *A. arius* collected from Vasco in all the seasons compared to those from Palolem.

3.4. Comet assay

The quantum of DNA damage in the form of % tail DNA is represented in Fig. 5. The % tail DNA was significantly high at Vasco in the cells of *A. arius* in all the seasons. The data also showed a high positive correlation ($R = 0.88$) between % MNi and % tail DNA in all the

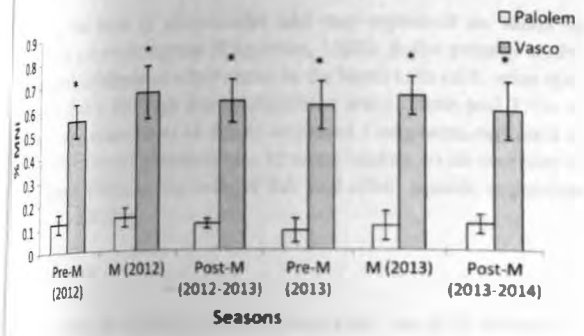

Fig. 4. Micronuclei (%) in the erythrocytes of *A. arius* (Mean ± S.D) collected from Vasco and Palolem in different seasons March 2012 to February 2014 (* $p < 0.05$). Pre-M = Pre-Monsoon; M = Monsoon; Post-M = Post-Monsoon.

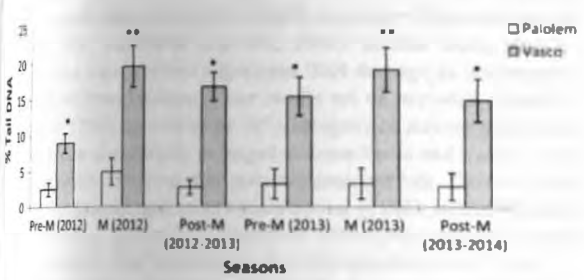

Fig. 5. DNA damage represented by Tail DNA (%) in erythrocytes of *A. arius* (Mean ± S.D) in different seasons from March 2012 to February 2014 (* $p < 0.05$; # $p < 0.01$). Pre-M = Pre-Monsoon; M = Monsoon; Post-M = Post-Monsoon.

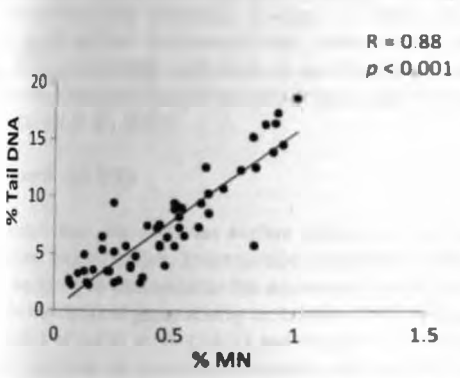

Fig. 6. Correlation analysis between % MNi and % Tail DNA in *A. arius*.

fish collected from the study sites (Fig. 6). DNA damage was found to be the highest during the monsoon sampling in 2012 as well as in 2013 ($p = 0.035$).

3.5 Principal components analysis

Ten components were extracted from the principal component analysis after varimax rotation of the factor axes which accounted for 87.22% of the total variance of the original variables. The first component which had a variance of 52.24% was characterized by high positive loadings of tissue and environmental concentrations of pollutants. The second component which explained a variance of 8.24% was characterized by loadings of physico-chemical parameters of surface and bottom water. The variables that were associated with the remaining components were the different seasons.

3.6. Multiple regression analysis

After factor scores were obtained from the ten components, the score values were used as independent variables and DNA damage (% Tail DNA) was used as the dependent variable due to the high correlation between the comet assay and micronucleus test in the present study (Table 2). The regressions of standardized DNA damage on component 1 was the most significant ($p < 0.0001$).

Table 2
Multiple regression model for predicting the DNA damage using factor scores obtained from principal components analysis.

Predictors	Coefficients	t-value	Significance (p)
Component 1	0.875 ± 0.015	16.91	< 0.0001
Component 2	−0.016 ± 0.015	−0.30	0.763
Component 3	0.048 ± 0.015	0.92	0.359
Component 4	0.089 ± 0.015	−1.72	0.089
Component 5	−0.170 ± 0.015	−3.29	0.052
Component 6	−0.060 ± 0.015	−1.16	0.248
Component 7	−0.056 ± 0.015	−1.07	0.287
Component 8	0.143 ± 0.015	0.76	0.058
Component 9	0.032 ± 0.015	0.61	0.542
Component 10	0.049 ± 0.015	0.94	0.347

$R^2 = 0.837$, Adjusted $R^2 = 0.810$

Table 3
Correlation coefficients (Pearsons) between DNA damage and levels of heavy metals in the tissues.

	TPH	Fe	Mn	Cu	Cd	Pb
Muscle	0.88***	0.84***	−0.23	0.60**	0.77***	0.80***
Gill	0.87***	0.87***	0.83***	0.43*	0.74***	0.76***
Liver	0.89***	0.86***	0.75***	0.39*	0.75***	0.81***

* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

3.7. Association between genotoxicity and pollutants

The correlation coefficients for the different tissues are given in Table 3. Highest coefficients were contributed by TPHs and Fe. Further, on comparison of the coefficients between tissues, differences between muscle and gill ($z = 0.25$, $p = 0.8$), muscle and liver ($z = -0.27$, $p = 0.78$) and gill and liver ($z = -0.52$, $p = 0.6$) for TPH was not significant. The same was observed for Fe ($z = -0.66$, $p = 0.5$; $z = -0.42$, $p = 0.6$; $z = 0.23$, $p = 0.81$), Cu ($z = 1.37$, $p = 0.17$; $z = 1.65$, $p = 0.09$; $z = 0.28$, $p = 0.77$), Cd ($z = 0.41$, $p = 0.68$; $z = 0.28$, $p = 0.77$; $z = -0.13$, $p = 0.89$) and Pb ($z = 0.6$, $p = 0.54$; $z = -0.7$, $p = 0.86$; $z = -0.77$, $p = 0.44$). Although the differences between gill and liver for Mn were not significant ($z = 1.26$, $p = 0.2$), it was significant between muscle and gill ($z = -8.35$, $p < 0.001$) and muscle and liver ($z = -7.09$, $p < 0.001$).

4. Discussions

The present study revealed that the water in Vasco contains high concentrations of TPHs and trace metals, which might have induced DNA damage, as evident by the MNi test and comet assay. This can lead to the deterioration in the health of the fish populations as well as decline in fish catches around this area and may pose a threat to the health and livelihood of the fishing communities at Vasco. Further, physico-chemical parameters and seasonality may also affect the accumulation of pollutants and subsequent DNA damage.

4.1. Micronucleus test

Several scientists have employed the micronucleus test to assess the genotoxicity of compounds released by industries into aquatic environments (Baršiene et al., 2004; Cavas and Ergene-Gozukara, 2005; Da Silva Souza and Fontanetti, 2006). Damaged chromosomes result from inefficient or incorrect DNA repair and/or from the presence of metals around the mitotic apparatus. This damage is expressed during cell

division in the form of micronuclei and may represent an index of accumulated genotoxic agents (Kligerman, 1982). In the present study, the increased incidence of micronuclei in the blood cells of *A. arius* may possibly be due to the high concentrations of trace metals and TPHs at Vasco. Another reason may be due to increased / long-term exposure to low concentrations of genotoxicants in water leading to an increase in the genotoxic effects in the cells of fish and other aquatic organisms (Mink et al., 2007).

4.2. Comet assay

DNA damage as measured by the comet assay has been linked to a wide spectrum of genotoxic and cytotoxic compounds, such as PAHs and trace metals (Lee and Steinert, 2003). The amount of the DNA in the tail region (tail DNA) is commonly used for quantifying DNA strand breakage and represents the most reliable parameter (Mitchelmore and Chipman, 1998; Kumaravel and Jha, 2006). In this study, the fish collected from Vasco showed significant DNA damage as compared to those collected from Palolem. These results are on par with a study by Sarkar et al. (2008) in which the DNA integrity in marine gastropods was found to be significantly damaged at Dona Paula and Vasco during the pre-monsoon, monsoon and post-monsoon periods. Chronic exposure to pollutants can lead to an accumulation of DNA strand breaks in aquatic organisms, such as fish, since their DNA-repair capacity is much lower compared to that of other species (Theodorakis et al., 1994).

DNA damage by xenobiotics occurs in three basic steps (Monserrat et al., 2007), wherein the first is the formation of adducts with toxic molecules. The next stage, secondary modifications of DNA, includes single- and double-strand breakage, changes in DNA repair, base oxidation and cross-links. Xenobiotics may induce these secondary modifications by way of ROS production. In the third stage, cells show altered function, which can lead to cell proliferation and consequently, cancer (Monserrat et al., 2007).

4.3. Trace metals and TPHs

Trace metals may settle on the surface sediments and can affect benthic feeders, such as catfish. Trace metals, especially Fe, Mn, Cd and Pb, which are found to contaminate the water and sediment at Vasco, could also be the cause of genotoxicity in *A. arius*. This is in agreement with the studies of Bellas et al. (2007) and Omar et al. (2012) which reported the genotoxic effects of trace metals, such as Cu, Zn, Fe, Mn and Pb, in marine and estuarine environments. The presence of high concentrations of these metals due to extensive mining activities and transportation of ores can be attributed to the stress caused in the fish. de Andrade et al. (2004) observed genotoxic damage measured as single-strand DNA breaks employing the comet assay in the tissues of mullet and sea catfish from polluted sites in Brazilian rivers and reported the association between the concentrations of hydrocarbons, trace metals, pH and water temperature and the levels of DNA damage. The multiple regression analysis conducted by us in the present study revealed that high levels of DNA damage are due to the accumulation of the pollutants from the environment. Physico-chemical parameters and seasonality also play a role in influencing DNA damage, but to a lesser extent. Trace metal accumulation in different tissues at high concentrations could occur due to various reasons. Trace metal accumulation in the liver could occur during metal detoxification in fish; it may occur in the muscle tissues because of absorption of residues through the intestinal walls, and it occurs in the gills due to metal complex formation with mucus on the gill lamellae (Taylor et al., 1985; du Preez et al., 1993). Particulate trace metals (nano-sized) may interact with fish or other organisms in four ways: 1) adsorption to the surface of cell, organ or whole body 2) cellular internalization 3) dissolution of ions from the NP and 4) mechanistic nano-effects, such as formation of reactive oxygen species (ROS) (Baker et al., 2014). Trace metals like cadmium and copper may also affect embryonic or larval stages and may cause increased mortality, altered body size as well as morphological deformities (Witeska et al., 2014). These findings can be compared with the results of Costa et al. (2011), in which the exposure to sediments contaminated by trace metals induced DNA fragmentation and clastogenesis in Senegalese soles.

The presence of TPHs in the tissues of the fish in the present study also indicate the presence of carcinogenic components in these hyrocarbons such as benzo[α]pyrene (B[α]P). B[α]P, a representative polycylic aromatic hydrocarbon (PAH) present in crude oil fractions, is reported to be converted at the cellular level to the ROS, diol-epoxide (BaPDE), which can form stable adduct with DNA, resulting in DNA strand breaks (Pisoni et al., 2004; Bihari and Fafandel, 2004). Balk et al. (2011) reported the presence of PAH metabolites in the bile of fish collected from the North Sea which is known to be polluted with petroleum hydrocarbons. These metabolites, besides being genotoxic can exert other toxic effects in the rest of the body (Aas et al., 2000) and may also affect the nutritive quality of fish tissue (Ansari et al., 2012).

In addition, although a significant association is seen between the pollutants and genotoxicity, it may also be noted that the presence of other contaminants in lesser concentrations may also influence the genotoxicity in these fish and can be determined by further studies using both field and laboratory based experiments.

5. Conclusion

Although the concentrations of contaminants in these fish are much below the permissible limits recommended by EC (2005), FAO (1983) and IAEA (2003), it may be noted that these fish are consumed throughout the year by the Goan population and may lead to subsequent bioaccumulation and genotoxicity in humans as well. The data for genotoxicity recorded in these fish can be used as reference values for future genotoxicity, biochemical or physiological studies at these sites. Laboratory based studies can be conducted to confirm the genotoxicity of these pollutants either singly or in association with each other and the exact pathways of genotoxic damage can be deduced as future work. As the industrial wastewaters in Goa are not properly processed and treated before release into the environment, a treatment method should be devised and enforced by the Government and Coast Guard. Pollution indicators and genotoxicity tests, combined with other physiological or biochemical parameters represent an essential tool for efficient monitoring of aquatic ecosystems in Goa.

Declaration of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2017.03.049.

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The Backwater Clam (*Meretrix casta*) as a bioindicator species for monitoring the pollution of an estuarine environment by genotoxic agents

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ABSTRACT

The coast of Goa receives anthropogenic stress through its major rivers, which carry mining wastes, including iron and manganese ores from upstream mining sites, and petroleum hydrocarbons from shipping activities. These contaminants show seasonal variation in concentration and may be bioaccumulated by fauna inhabiting these waters. These fauna, including the bivalve molluscs, are particularly at risk due to these insults. In the present study, the use of the backwater clam, *Meretrix casta* (Chemnitz), as a bioindicator species was evaluated, comparing two sites (Vasco and Palolem) on the Goan coast. DNA damage was assessed in the gill cells using the micronucleus and comet assays; physiological condition was determined from the condition index. These values were tested for correlations with the concentrations of total petroleum hydrocarbons and trace metals in the whole soft tissues and with the physico-chemical parameters of water from these sites. Specimens collected from Vasco showed high incidence of micronuclei and % tail DNA and a low condition index ratio compared to those from Palolem, which correlates with the higher level of pollutants in the bivalves the former site. We believe that *M. casta* is a suitable species for biomonitoring studies of this type.

Introduction

Biomonitors are organisms which can be used to detect environmental stressors [1]. Any organism can be considered as a bioindicator, depending upon its sensitivity [2]. Although exposure to these stressors can be compensated by repair mechanisms, signs of toxicity will most likely be evident at the upper limits of tolerance. Bioindicators are key for assessing the level of environmental pollution, especially at sites of anthropogenic stress.

Molluscs, particularly bivalves, are popular "sentinels" for detecting environmental contamination [3], because of their association with the marine sediment, filter-feeding habit, and ability to bioaccumulate contaminants. Bivalve species such as *Mytilus galloprovincialis* and *Ruditapes philippinarium* are routinely used for these programs, either as natural populations or cultured and transplanted to the locations under study [4–7]. Two genotoxicity tests, the micronucleus test (MN) and the comet assay are routinely used as biomarkers for monitoring aquatic pollution by genotoxic contaminants. These tests are of significant toxicological importance since they can detect DNA damage caused by exposure to pollution [8–10]. Further, these tests, when combined with other physiological and biochemical biomarkers, can give a wider perspective of the pollution status of water bodies.

The potential of using clams as sentinel species has been demonstrated by Fernández-Tajes et al. [11], who used three species of bivalves (*Venerupis pullastra, Cerastoderma edule* and *Mytilus galloprovincialis*) for biomonitoring an estuary in northwest Spain. DNA damage (comet assay) was correlated with pollution loads; clams and cockles were more sensitive to the effects of pollution. In another study, Jebali et al. [12] reported increased DNA damage in the clam, *Ruditapes decussatus*, collected from polluted sites along the coast of Tunisia. A multibiomarker approach using genotoxicity assays along with physiological and biochemical assays could be used to assess the quality of water and sediment at polluted sites [13].

According to the US EPA, shellfish can take up these metals by several mechanisms: (1) ingestion of particulate substances from suspended material; (2) ingestion of food materials that have acquired these metals; (3) uptake by exchange onto mucous sheets; (4) incorporation of these metal ions into physiologically important systems; and (5) formation of metal complexes with other organic molecules [14].

Meretrix casta (Chemnitz), which is selected for the present study, commonly called the backwater or estuarine clam, is usually found in the backwaters or estuaries of both the east and west coast of India [15]. It is a commercially important bivalve species that is consumed as a local delicacy in many parts along the coast of Goa and is available throughout the year. A few toxicity studies are available on *M. casta*.

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Balasubramanian [16] observed changes in the condition
l. casta exposed to water-soluble fractions of diesel, engine
wait crude oil for a period of 30 d. *M. casta* exposed to
es of γ-radiation exhibited single-strand breaks in DNA of
cytes, as measured by the comet assay [17].
ng to the Indian Bureau of Mines of the Ministry of Mines
nt of India), Goa accounts for 18% of the iron ore and 3% of
nese ore in India [18,19]. The major rivers of Goa are con-
with these metals, predominantly due to mining of iron and
ore deposits [20–22]. The metals are then carried down-
the estuarine regions [23] and may either circulate in the
mn or be deposited on the surface sediment and interact with
ated benthic fauna. A number of reports are available on the
tions of iron and manganese prevailing in the estuaries of Goa

resent study aims to assess the use of a bivalve as a bioindi-
cies to monitor the pollution status of estuarine waters. The
es were selected based on available reports. Vasco, at the
f the Zuari river, is reported as a polluted estuary [27,28];
close to the Talpona river, is a relatively pristine environment

rials and methods

dy sites

co was selected as the polluted site (Fig. 1), based on available
re [27,29]. Ores of iron and manganese are transported to off-
arges via the Zuari river and thus the water and sediment at
are contaminated with metals, petroleum hydrocarbons, and
lorinated biphenyls. Palolem was selected as the reference site
its pristine nature and lack of industrial activity at this site [29].

Fig. 1. Study area, showing the sampling sites at Vasco and Palolem.

2.2. Water sampling

Samples were collected from three sampling stations at each site during the pre-monsoon, monsoon, and post-monsoon periods from March 2012 to February 2014. The temperature, pH, and salinity of the water samples were recorded onsite with the help of an automated water analysis kit (Labtronics, India). Water samples for dissolved oxygen were taken in amber-coloured bottles (300 ml), fixed with Winkler's solution, and analyzed in the lab.

2.3. Bivalve collection

Meretrix casta (Estuarine backwater clam) was selected for the present study for its commercial value and availability in the Goan estuaries throughout the year. *M. casta* are moderately large bivalves with an average length of about 48 mm. They are identified by their thick shell, brownish appearance and anterior umbo (Fig. 2). Adult bivalves (n = 50) were collected twice during each season from each site with the help of skilled local fishermen. They were stored in a bucket with water from the study site, transported alive, and immediately processed upon arrival at the laboratory. Fifteen bivalves were used for the MN test and comet assay (detailed below). Another 10 bivalves were used for estimation of condition index. The remaining bivalves were used for the determination of petroleum hydrocarbons and trace metals.

2.4. Quality assurance and quality control

The appropriate quality assurance methods of sample preparation, handling, and preservation were carried out in accordance with US EPA procedures. All chemicals used were of analytical grade from Himedia (Himedia, India) unless specified otherwise.

2.5. Analysis of samples

The bivalves were dissected open and their gill tissues were collected and cleaned thoroughly prior to the analyses.

2.5.1. Micronucleus test

The micronucleus (MN) test was performed following the protocol outlined by Barsiene et al. [30]. A portion of the gill tissue was placed in a drop of methanol acetic acid mixture (3:1) on a clean glass slide. The gills were then gently nipped with tweezers for a few minutes and the resulting cell suspension was then smeared and air-dried. The smears were fixed in methanol for 10 min, stained with 5% Giemsa for 15 min, and allowed to dry. Five slides were prepared for each bivalve and were analyzed blind from coded slides. The frequency of micronuclei (MN) was recorded by scoring 1000 intact cells per slide at 1000× magnification using an Olympus BX53 microscope. MN were identified according to the following criteria: (1) spherical or ovoid-shaped extra nuclear bodies in the cytoplasm; (2) diameter 1/3–1/20 of the main nucleus; (3) non-refractory bodies; (4) colour texture and optical features resembling those of the nucleus; and (5) the bodies completely separated from the main nucleus.

2.5.2. Single cell gel electrophoresis (comet assay)

The comet assay was carried out as per the protocol of Lee and Steinert [31]. Gill tissue (0.1 g) was homogenized gently with phosphate buffer saline (pH 7.4) and the resulting cell suspension was passed through a muslin cloth to filter tissue debris. Prior to analysis, the cell count and cell viability of the suspension were checked (trypan blue dye exclusion) to ensure that there were enough living cells to perform the assay. Samples showing more than 90% viability and a cell count > 10^6 cells/ml were used for the tests. The cell suspension was embedded in low-melting agarose (0.5% LMA) on frosted microscopic slides precoated with 1% normal-melting agarose (NMA). The cells

Fig. 2. Backwater clam, *Meretrix casta*, actively feeding in water.

...re then lysed by placing the slides in cold lysing solution (2.5 M ...Cl, 100 mM Na_2EDTA, 10 mM Tris, 10% DMSO and 1% Triton-X, pH ...) at 4 °C, overnight. Following lysis, the slides were placed in un...ding buffer (electrophoresis buffer, pH 10) for 20 min to allow the ...A to unwind. Electrophoresis was then performed for 30 min at ...74 V/cm (Biorad electrophoresis unit).

The slides were placed in neutralization buffer (400 mM Tris base, ...7.5) for 5 min. The gel containing DNA was stained with ethidium ...omide and examined using a fluorescence microscope (Olympus ...53) with a green filter at 200× magnification. All the above men...ned steps were performed in dim light and at 4 °C to prevent photo...diction of DNA. 5 slides were prepared for each bivalve and 100 ...domly selected non-overlapping cells from each slide were screened ...d analyzed with the help of the computer software, CASP [32], and ...e % tail DNA was recorded. The % tail DNA is the amount of DNA (in ...cent) present in the tail of the "comet" and is used as a measure of ...A damage [33].

...3 Condition index

Bivalves were cleaned, dissected and the soft tissue was carefully ...arated from the hard shells. Both the soft tissue and the shells were ...ed in an oven at 60 °C overnight to determine their dry weights ...l. Condition index (CI) was then calculated as follows:

$$\text{...ndition index} = \frac{\text{Dry softtissue weight(g)}}{\text{Dry shell weight(g)}} \times 100$$

...4 Total petroleum hydrocarbon (TPH) analysis

Whole soft tissues were pooled (~10 g in triplicates) and homo...ized with hexane in a Potter-Elvehjem homogenizer and were then ...icated in a sonicator water bath. The samples were dried by passing ...ough a column of anhydrous sodium sulphate and made to a final ...ume of 25 ml with hexane. The extracted samples were placed in ...rtz cuvettes and the concentration of total petroleum hydrocarbons ...e analyzed using a spectrofluorophotometer (Shimadzu RF-5301 PC) ...h synchronous excitation at 310 nm and emission at 360 nm. The ...ified reference material for TPH estimation was Bombay High crude ... and was used to prepare the calibration curve. The values were expressed as μg/g of wet tissue [35].

2.5.5. Trace metal analysis

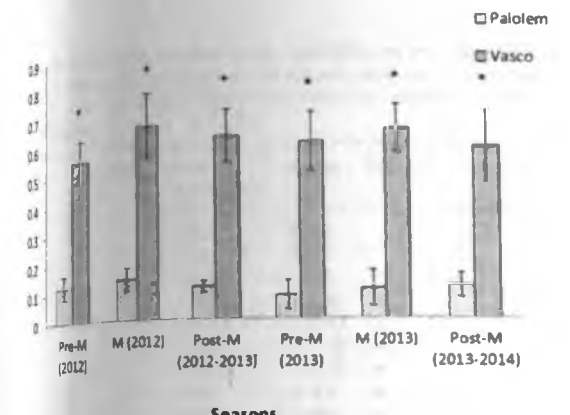
Whole tissue samples (~2 g in triplicates) were digested in 10 ml of 14 M nitric acid, 13 M perchloric acid and 5 ml distilled water in a Teflon beaker, according to Begum et al. [36]. The samples were heated at 150 °C for 2 h on a hot plate. The sample was then transferred to a 100 ml volumetric flask and diluted to 50 ml with distilled water. The concentrations of iron (Fe), manganese (Mn), copper (Cu), cadmium (Cd), and lead (Pb) were determined from this sample by Flame Atomic Absorption Spectrophotometry (Shimadzu). Results were expressed as μg/g of wet tissue. "TraceCERT certified reference metals for AAS (Sigma)" were used for trace metal estimation.

2.6. Statistical analyses

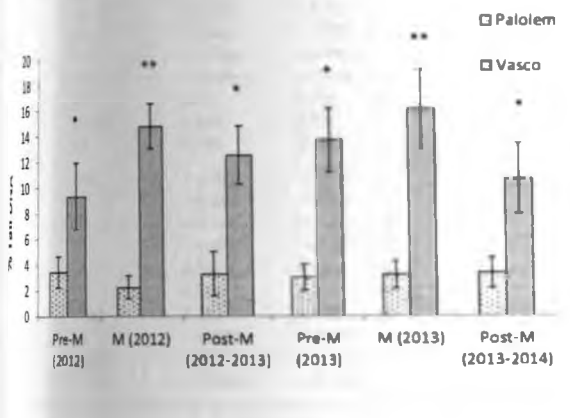
Statistical analysis was performed using IBM© SPSS 23 statistical software package. Data of MN test and % tail DNA were arc sine transformed and that of CI, tissue concentrations of pollutants and physico-chemical parameters of water were log transformed to improve linearity and were tested for normality and homogeneity using the Shapiro-Wilk test and Levene's test, respectively, prior to subsequent analyses. Two-way analysis of variance (ANOVA) was used to check the influence of sites and seasons on MN, % Tail DNA and CI. The significance of the data of MN test, comet assay and condition index between the sites was analyzed employing a post hoc Dunnet's test. A multiple regression model was used to evaluate the factors affecting DNA damage and CI. Pearson's correlation analyses were done between (1) MN test and the comet assay; (2) MN test and CI; and (3) comet assay and CI. The results were regarded as statistically significant at $p < 0.001$, 0.01, and 0.05.

3. Results

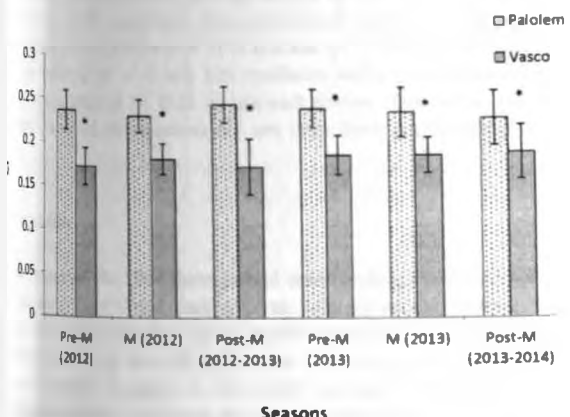
The bivalves collected from Vasco in all seasons had significantly high DNA damage, in the form of MN ($p < 0.05$) compared to those from Palolem (Fig. 3). Also, a significant increase in % tail DNA was observed in the clams collected from Vasco (Fig. 4). The condition



3 Micronuclei (%) in the gill cells of *M. casta* (Mean ± S.D) collected from Vasco Palolem in different seasons March 2012 to February 2014 ($^{*}p < 0.05$). *Note: Pre-M: nsoon; M: Monsoon, Post-M: Post-monsoon*



4. Tail DNA (%) in the gill cells of *M. casta* (Mean ± S.D) collected from Vasco and Palolem in different seasons March 2012 to February 2014 ($^{*}p < 0.05$; $^{**}p < 0.01$). *te: Pre-M: Pre-monsoon; M: Monsoon, Post-M: Post-monsoon*



5. CI of *M. casta* (Mean ± S.D) collected from Vasco and Palolem in different sea- March 2012 to February 2014 ($^{*}p < 0.05$; $^{**}p < 0.01$). *Note: Pre-M: Pre monsoon; lonsoon, Post-M: Post-monsoon.*

ex of all the bivalves collected from Vasco was also found to be ificantly low ($p < 0.05$, Fig. 5).

The physico-chemical parameters of water and the concentrations of l and trace metals in the soft tissues of *M. casta* are given in S1 and espectively (Supplementary data). There were no significant var- ons in the water parameters throughout the seasons and sites. ever, the concentrations of TPH in the soft tissues of the bivalves collected from Vasco were significantly high compared to those collected from Palolem in all seasons ($p < 0.05$). The metals Fe, Mn, Cd, and Pb were also significantly high in most seasons through the study period. Cu was found to be significantly high only in the monsoon season of 2013.

The results of the two-way ANOVA using MN, % Tail DNA or CI as the dependent variable, with sites and seasons as the independent variables, showed an interesting trend. The variance between MN and sites was highly significant ($F = 92.15$, $p < 0.001$) whereas between MN and seasons was not significant ($F = 0.35$, $p = 0.35$). Similarly, the variance between % tail DNA and sites and that between CI and sites was also highly significant ($F = 113.22$, $p < 0.001$ and $F = 84.49$, $p < 0.001$ respectively). The variance between both % tail DNA and CI when individually compared with seasons was not significant ($F = 0.32$, $p < 0.4$ and $F = 0.41$, $p < 0.16$ respectively). Additionally, the influences of both season and site on MN, % tail DNA, or CI were non-significant ($F = 1.28$, $p < 0.09$; $F = 1.12$, $p < 0.11$ and $F = 0.84$, $p < 0.15$ respectively). Therefore, DNA damage and condition index depend on the sites and not on the season.

Based on the correlation matrix between the genotoxicity and condition index parameters, a positive correlation was obtained between the frequency of MN and % tail DNA ($R = 0.85$, $p < 0.001$), also represented as scatter plots (Fig. 6). A significant negative correlation was observed between the MN test and CI ($R = -0.72$,

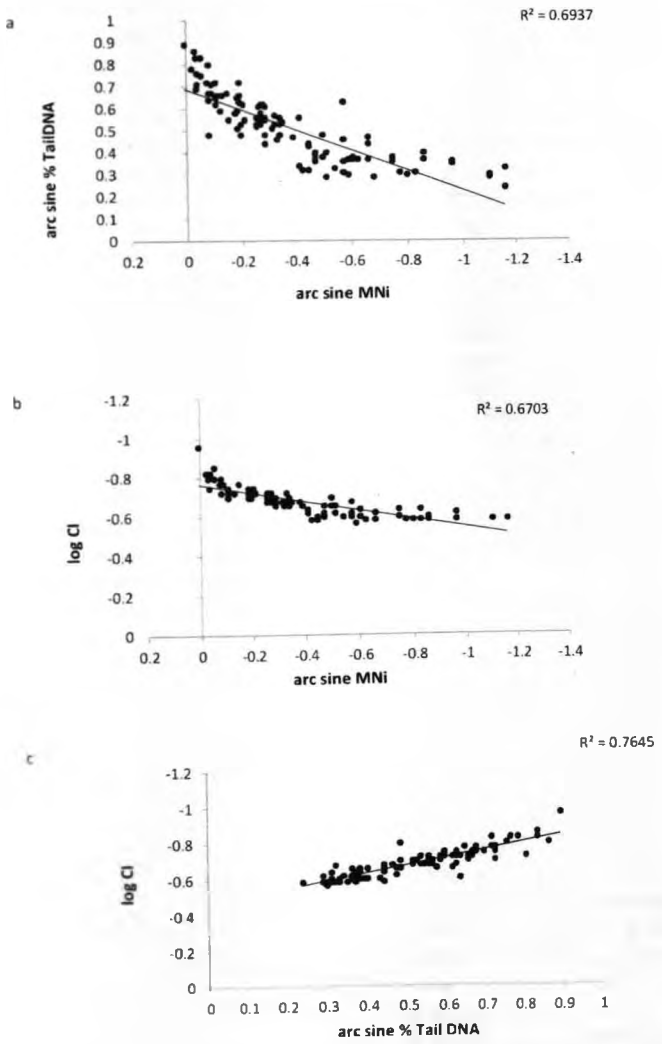


Fig. 6. Correlation analysis between **a.** log MN and arc sine % Tail DNA, **b.** log MN and log CI and **c.** arc sine % Tail DNA and log CI in *M. casta*.

...e regression model using micronuclei (MN), % tail DNA, and condition index (CI) ...pendent variables and concentrations of TPH and trace metals in tissues, tempera... temp), pH, salinity, and dissolved oxygen (DO) of water as independent variables in ...ent combinations (**$p < 0.01$, *$p < 0.05$)

...pendent ...iable	Independent variable	Beta Coefficients	t value	Significance	R square
...)	TPH	0.631	2.236		0.71
	Fe	0.431	2.383		
	Mn	0.249	2.244		
	Cu	−0.051	−0.481	ns	
	Cd	−0.102	−0.615	ns	
	Pb	−0.201	−1.410	ns	
	Temp	−0.020	−0.272	ns	
	pH	0.017	0.193	ns	
	Salinity	0.151	1.434	ns	
	DO	−0.012	−0.096	ns	
...tail DNA	TPH	0.869	2.895		0.62
	Fe	0.184	0.955	ns	
	Mn	0.453	3.846		
	Cu	−0.079	−0.705	ns	
	Cd	−0.244	−1.388	ns	
	Pb	−0.090	−0.594	ns	
	Temp	−0.025	−0.319	ns	
	pH	0.111	1.157	ns	
	Salinity	0.264	2.355		
	DO	0.038	0.282	ns	
...	TPH	−1.029	−3.115		0.54
	Fe	−0.183	−0.865	ns	
	Mn	−0.185	−1.429	ns	
	Cu	−0.053	−0.429	ns	
	Cd	0.231	1.193	ns	
	Pb	0.206	1.237	ns	
	Temp	0.042	0.493	ns	
	pH	−0.078	−0.745	ns	
	Salinity	−0.175	−1.423	ns	
	DO	−0.106	−0.709	ns	

< 0.01) as well as between the comet assay and CI (R = −0.85, < 0.001).

In the multiple regression model (Table 1), the induction of MN was ...ificantly affected by the concentrations of TPH, Fe, and Mn in the ...ues ($p < 0.05$). Similarly, the incidence of % tail DNA was affected ... the tissue concentrations of TPH and Mn ($p < 0.05$, $p < 0.01$) and ... water salinity ($p < 0.05$). The condition index was influenced only ... the concentration of TPH in the soft tissues. The other variables ...ysico-chemical parameters) did not contribute significantly to this ...del.

... Discussion

We examined the DNA damage and deteriorating condition index of ...eretrix casta from Vasco compared to Palolem across seasons. In all ...asons, *M. casta* collected from Vasco showed a significantly high ...quency of MN in the gill cells. This is in agreement with several ...orts of genetic damage in the bivalves exposed to pollutants in...luding trace metals, various hydrocarbons and biphenyls, such as that ...Schiedek et al. [37], who reported a significantly high frequency of ...N in blue mussels (*M. edulis*) from a location polluted with metals, ...BHs, and organochlorines in the Baltic Sea. Barsiene et al. [38] sam...led blue mussels (*Mytilus* spp.) from different zones in the Baltic Sea ...nd reported a high frequency of MN in the gill cells of mussels from ...ighly polluted areas. In another study, *M. edulis* collected from the ...altic Sea after an accidental oil spill showed significant elevation in ...he frequency of MN compared to those collected before the oil spill ...39].

The presence of these MN has been attributed to the presence of ...enobiotics in the environment. MN, which arise due to lagging chro...osomal fragments or a whole chromosome, do not get incorporated during cell division and persist as a small body (micronucleus) in proximity to the main nucleus; thus, this assay is useful for any proliferating cell population [40]. The lagging chromosomal fragments/chromosomes arise as a consequence of long-term exposure associated with an accumulated effect of genotoxic or clastogenic agents which either directly affect the DNA or interfere with mitotic processes [13,30,41].

A significant increase in the % tail DNA was observed from the clams collected from Vasco (Fig. 3). This indicates the presence of a high level of DNA-damaging agents, probably trace metals and various hydrocarbons, which will lead to the loss of DNA integrity through DNA strand breaks [13]. Our observation is in agreement with that of Sarkar et al. [42] who reported a decrease in DNA integrity in the gastropod, *Cronia contracta*, from Vasco and were reported to be attributed to exposure to polycyclic aromatic hydrocarbons and metals such as Pb, Cu, Cd, Fe, and Mn. In another study, Sarkar et al. [27] reported a decrease in DNA integrity of another gastropod, *Morula granulata* from Hollant, which is located close to Vasco. Certain petroleum hydrocarbons constituents, such as benzo[*a*]pyrene (a polycyclic aromatic hydrocarbon), when absorbed by the organism, can be converted to a reactive species (dihydrodiol epoxide), and can form a stable adduct with DNA [43], leading to changes in the conformation and functioning of the DNA and to carcinogenesis [44,45].

We also observed a significant positive correlation between the frequencies of MN and % tail DNA. The MN test and the comet assay reflect different forms of environmental stress. The comet assay is able to detect repairable DNA damage such as DNA strand breakages, whereas the MN test detects more persistent DNA damage such as chromosomal breaks which are more difficult to repair [46,47]. The combination of bioaccumulated contaminants in the tissues of the bivalves could therefore lead to formation of complexes with metabolites, reactive oxygen species and subsequently DNA damage which could initially induce DNA strand breaks; whereas prolonged exposure would lead to more severe damage such as MN formation.

A significant negative correlation was observed between the MN test and CI as well as between the % tail DNA and CI. Langston et al. [48] reported a significant negative correlation between condition index and tissue levels of environmental contaminants of *Littorina littorea, Mytilus edulis* and *Cerastoderma edule* in a river system (Milford Haven Waterway) of Wales, UK. The decrease of physiological health may be attributed to altered DNA function which subsequently results in altered protein function that is ultimately required for normal physiological processes. The physiological state of the bivalve can also lead to changes in its feeding activity thereby altering its life cycle as well [49]. Another reason for the decrease in the CI ratio could possibly be the survival adaptive response of *M. casta*, reducing the filtration rate or closing the shell on exposure to contaminants [50].

Meretrix casta collected from Vasco also showed significant concentrations of total petroleum hydrocarbons and trace metals compared to ones collected from Palolem. These observations also support our previous findings of TPH and trace metals in the tissues of an edible finfish, *Arius arius* from Vasco [10]. The presence of these pollutants, especially TPH, Fe and Mn, in the tissues is also associated with genotoxicity in these organisms. The reason for this association could be the intensity of mining and harbor activities at Vasco, as mentioned earlier. Trace metals, which may be genotoxic when accumulated at a high concentration in the tissues of aquatic organisms, can have a profound effect on the integrity of DNA leading to single- or double-strand breaks [4]. Trace metals can also cause the formation of reactive oxygen species (ROS) [51] and can therefore indirectly cause damage to DNA, alter proteins and induce lipid peroxidation, resulting in decreased nutritive value and disease following consumption [52–54]. Another consequence of bioaccumulation of metals is the effect on the physiological well-being of the organism which, in turn, also affects its meat quality [55,56]. The presence of petroleum hydrocarbons in the bivalves also induce genotoxicity by forming metabolites and interact

DNA either directly by forming DNA adducts or indirectly by the ation of ROS [43,57,58]. The presence of TPH in the present study associated with a reduced condition index and may thus affect quality and palatability [35].

Seasonality plays an important role in the bioaccumulation of en- mental contaminants and in turn may affect DNA damage -62]. However, based on the two-way ANOVA in our present study, nality does not seem to affect DNA damage. This may be due to mations in the physico-chemical parameters throughout the sea- , which play a major influence in contaminant uptake, or due to sistence of the contaminants in the environment as well as in the sues of the clam. Our results are in agreement with that of Bocchetti al. [63], in which they did not observe significant seasonal variation DNA damage (particularly MN) in clams and mussels collected from Adriatic Sea. Our results are also comparable with that of Singh and ut [64], who reported that changes in environmental conditions do affect DNA damage in *M. edulis* exposed to waters with different nities. This could be due to species-specific mechanisms as both ms and mussels are well adapted to the intertidal environment [65]. Further studies are required to understand the mechanisms of sociation of DNA damage and seasonal variations. On the other hand, nificant variation in DNA damage and contaminant accumulation as observed between the sites in the present study. This can be at- buted to the level of anthropogenic activities occurring at both the s. Site selection thus plays an important role in which biological fects can be appropriately compared and can provide a baseline data r future work [66,67].

Conclusion

Based on the observations of DNA damage, physiological condition dex, and its occurrence despite the presence of pollution, *M. casta* is a ood candidate for biomonitoring studies in estuarine ecosystems. The ll test and comet assay can be easily employed for analyzing geno- icity. Further analyses of the water column and sediment could plement our findings and provide a broader perspective of the erall health at these sites since bivalves are an economically im- rtant species. Further, the stress caused by DNA damage leads to eriorated physical condition and could also lead to a significant cline in natural populations. Regular monitoring of estuaries for xe- biotic pollutants is needed.

nflicts of interest

The authors report no conflicts of interest. The authors alone are sponsible for the content and writing of the paper.

knowledgements

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ppendix A. Supplementary data

Supplementary data associated with this article can be found, in the line version, at http://dx.doi.org/10.1016/j.mrgentox.2017.11.001.

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Genotoxic and Biochemical Biomarker Responses in *Meretrix Casta* Exposed to Environmentally Relevant Concentrations of Cadmium

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Abstract

Cadmium (Cd) enters estuarine water by surface runoffs from mines, phosphate fertilizers from agricultural fields and other anthropogenic sources which may pose a threat to the fauna inhabiting these waters. Bivalve molluscs which are a source of seafood may accumulate Cd leading to deteriorated health of the organisms and may also cause various health consequences in man. The present study was carried out to assess the toxicity of Cd in the estuarine backwater clam, *Meretrix casta*. In the experimental setup, the bivalves were exposed to three environmentally relevant concentrations of $CdCl_2$ (0.75 µg/L, 1.5 µg/L, 3 µg/L) for a period of 15 days. Genotoxicity tests [Micronucleus Test (MN) and comet assay], oxidative stress parameters [Catalase Assay (CAT) and Lipid Peroxidation Assay (MDA)], neurotoxicity test [Acetylcholinesterase Assay (AChE)] and physical condition (condition index) were employed to evaluate the effects of Cd in *M. casta*. A dose-dependent increase of DNA damage was seen at all the concentrations of Cd. Catalase activity was not significantly changed at the lowest concentration compared to the control, but increased significantly at the higher concentrations. Lipid peroxidation was found to be significantly increased at all the concentrations of Cd. However, the levels of AChE were found to decrease in a dose-dependent manner. The condition index ratio was also found to be lowered with increasing dose. DNA damage was highly correlated with oxidative stress suggesting the mechanism of action of Cd on DNA. Oxidative stress was negatively correlated with AChE and may possibly be a contributing factor to neurotoxicity. *M. casta* can therefore be used as a potential sentinel species for monitoring the Cd in the estuarine environment using these biomarkers.

Introduction

Cadmium (Cd) occurs naturally in sedimentary rocks and soils in the environment and is also a constituent of zinc, lead and copper ores. It is used extensively in various applications such as anticorrosive agents, stabilizers in PVC products, in pigments, a neutron-absorber in nuclear power plants and the manufacture of nickel-cadmium batteries. It is also present in phosphate fertilizers (1). Cd can therefore be released into the aquatic environment from sources such as rainwater runoffs from metal mining sites, mine drainage water, phosphate fertilizers, sewage treatment plants landfills and hazardous waste sites (2,3). Cd is known to be implicated in carcinogenesis either through oxidative stress or inhibition of DNA repair processes (4). The Environmental Protection Agency has thus classified Cd as a Group B1 carcinogen and is considered to be a probable human carcinogen (5). The toxicity of Cd is well documented in plants and is known to affect various important processes (6). In animal models such as fish, acute and sub-chronic exposure to Cd leads to alterations of gill epithelium, liver and kidneys and also affects enzymes such as acetyl cholinesterase (7,8). In molluscs, Cd exposure results in reduction of growth rate and mortality due to impairment of several metabolic functions (9,10). Several studies have also reported the genotoxicity of Cd in various animal models (11-15). The effect of Cd on DNA may be indirect, via the action of reactive oxygen species thus leading to oxidative DNA damage (16). Further, metal-induced genotoxicity is predominantly due to the inhibition of the DNA repair process (17).

Molluscs, particularly bivalves have been popularly used as "sentinels" to detect pollution caused by a wide array of contaminants in the environment (18). The advantage of using bivalves is due to their intimate association with the sediment, filter- or suspension-feeding habit and their ability to bioaccumulate various contaminants. Bivalves can selectively concentrate metal ions several hundred times from their surrounding water by several mechanisms such as the ingestion of particulate substances from suspended material, ingestion of food material that have acquired these metals, uptake by exchange onto mucous sheets of siphons and gills resulting in their incorporation into important physiological systems and formation of metal complexes with other organic molecules within the body (19). Cd, along with other hazardous metals is known to bioaccumulate in the tissues of bivalve molluscs which can pose a serious threat to the seafood consumers (20,21). Cd also affects the early development of bivalves causing growth abnormalities and reduced survival (22). The International Agency for Research on Cancer has reported that regular consumers of bivalve molluscs are estimated to have weekly dietary cadmium exposures of 4.6 µg/kg of body weight (3).

In the present study, *Meretrix casta* (Chemnitz) commonly called the backwater or estuarine clam, was selected due to its occurrence in the backwaters or estuaries of both the east and west coast of India (23). It is consumed as local seafood in many parts along the coast of India and is available throughout the year. Despite its consumption, few studies are available on the toxicity of various contaminants that predominantly occur

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these regions in *M. casta*. The presence of Cd in the waters ong the coast of Goa may be attributed to the discharge of fluents from agrochemical industries (24). Oysters *(Crassostea*) collected from a polluted estuary in Goa were found to ve high concentrations of Cd in their soft tissues which are pendent on the speciation of Cd in the water and sediment 5,26). High concentrations of Cd were also observed in oysters *madrasensis, C. gryphoides and Saccostrea cucullata)* collected om three different polluted sites in Goa and were found to be nsistently high in all the seasons (27).

Materials and Methods

Quality Assurance and Quality Control

The appropriate quality assurance methods of sample reparation, handling and preservation were carried out in ccordance with US EPA procedures. All chemicals used were of nalytical grade from Himedia (Himedia, India) unless specified herwise.

Maintenance of *Meretrix casta*

Meretrix casta (Estuarine backwater clam) was selected r the present study as it is consumed by a majority of the astal population and also due to its availability in the Goan stuaries throughout the year. The bivalves (both sexes) were llected from the intertidal zone with the help of skilled local shermen from Palolem, a pristine location in Goa. This site is clean, pristine beach with no known industrial activity or nthropogenic stress (28,29). They were stored in a bucket with ater from the study site and transported alive to the laboratory. They were then allowed to acclimatize in ordinary seawater from alolem for 30 days. The water conditions were maintained as llows: temperature 25°C, pH 7.5, salinity 25 ppt, dissolved ygen 7.5 mg/L. The water was changed once daily to reduce cal contamination.

Treatment Schedule

Bivalves were distributed in groups, each containing 0 individuals and were used for dose-response studies. Concentrations of $CdCl_2$ were selected based on the 96h LC50 alues in *M. meretrix* (30) and the environmental levels along the oan coast (31). Accordingly, three sub-lethal concentrations of $CdCl_2$ (0.75 µg/L, 1.5 µg/L and 3 µg/L) were selected and were posed to the bivalve groups for a period of 15 days. A group of valves was maintained in parallel without any Cd treatment and rved as the negative control.

Analysis of Samples

The bivalves were dissected open, their gill and muscle ssues were cleaned thoroughly prior to the genotoxicity and ochemical analyses.

Cell viability

Prior to the comet assay and micronucleus test, the cell unt and cell viability of the peripheral blood were checked to nsure that there were enough living cells to perform the assay mploying trypan blue dye exclusion test. The samples showing more than 90% viability and a cell count of a minimum of 10^6 cells/ml were used for the tests.

Micronucleus Test

The Micronucleus (MN) test was performed following the protocol outlined by Baršiene et al. (32). A portion of the gill tissue was placed in a drop of methanol acetic acid mixture (3:1) on a clean glass slide. This tissue was then gently nipped with tweezers for a few minutes and the resulting cell suspension was then smeared and air-dried. The smears were fixed in methanol for 10 min, stained with 5% Giemsa for 15 mins and allowed to dry. The frequency of Micronuclei (MNi) was recorded by scoring 2,000 intact cells per bivalve at 1000x magnification using an Olympus BX53 trinocular research microscope. Micronuclei (MNi) were identified according to the following criteria: (1) spherical or ovoid-shaped extra nuclear bodies in the cytoplasm (2) a diameter of 1/3 - 1/20 of the main nucleus (3) non-refractory bodies (4) colour texture and optical features resembling those of the nucleus, and (5) the bodies completely separated from the main nucleus.

Single Cell Gel Electrophoresis (Comet Assay)

The comet assay was carried out as per Lee and Steinert (33). All steps were carried out in dim light to prevent photo-oxidation of DNA. Gill tissue (0.1g) was homogenized gently with phosphate buffer saline (pH 7.4) and the resulting cell suspension was passed through a muslin cloth to filter out tissue debris. This cell suspension was then embedded in Low Melting Agarose (LMA) on frosted microscopic slides. The cells were then lysed by placing the slides in a cold lysing solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, 10% DMSO and 1% Triton-X pH 10) at 4°C, overnight. Following lysis the slides were placed in unwinding buffer (electrophoresis buffer, pH 10) for 15-20 min to allow the DNA to unwind. Electrophoresis was then performed for 30 min at 300 mA, 25 V (Biorad electrophoresis unit). The slides were placed in neutralization buffer (400 mM Tris base, pH 7.5) for 5 min. The gel containing DNA was stained with ethidium bromide and examined using a fluorescence microscope (Olympus BX53) with a green filter at 200x magnification. Randomly selected non-overlapping cells were screened and their comets were analyzed with the help of computer software, CASP (34) and the % tail DNA was recorded. The % tail DNA is the amount of DNA (in percent) present in the tail of the "comet" and is used as a measure of DNA damage (35).

Catalase Assay

Bivalves were dissected and their whole soft bodies were collected and homogenized in 50 mM of Tris buffer (pH 7.4) containing 0.3 M sucrose and 1 mM EDTA. This suspension was then centrifuged at 10,000 xg for 20 min at 4°C and the supernatant was collected. Catalase activity was carried as per Aebi (36) based on the decrease in absorbance of the test sample by the decomposition of H_2O_2. The reaction mixture consisted of 13.2 mM H_2O_2 in 50 mM phosphate buffer (pH 7.0) and 0.1 ml of the homogenate. The reduction in absorbance was measured at 240 nm using a multiwall plate reader (Analytical Technologies

...al) at 25°C over 3 minutes. Total protein concentration was ...easured by Bradford's method (37). The activity of Catalase ...AT) was expressed as µmol H_2O_2 $^{-1}$min^{-1}mg protein.

...cetylcholinesterase Assay

The Acetylcholinesterase (AChE) activity in whole soft ...odies of bivalves was determined using the Ellman et al. (38) ...ith modifications as described by Galloway et al. (39). Briefly, ...0 µL of sample homogenate was incubated in microtitre plates ...ith 150 µl DTNB (270 µM in 50 mM sodium phosphate pH ...4) at 25°C for 5 min. The enzyme activity was initiated by the ...ddition of 3 mM acetylthiocholine iodide and the absorbance ...as measured at 412 nm. The activity of AChE was expressed as ...mol thiocholine^{-1}min^{-1}mgprotein.

...Malondialdehyde Assay

The Malondialdehyde (MDA) assay which is used to ...est lipid peroxidation in the whole soft bodies of bivalves was ...arried out using a commercial kit (North West Life Science ...pecialities- NWK-MDA01). The assay is based on the reaction ...f MDA with Thiobarbituric Acid (TBA) forming a pink coloured ...DA-TBA_2 adduct that absorbs strongly at 532 nm. Butylated ...ydroxytoluene (BHT) and EDTA are also added to the reaction ...ixture containing the sample homogenate to minimize oxidation ...f lipids. The activity of MDA was expressed as nmol MDA^{1}min ...mgprotein.

...Condition Index

Bivalves were cleaned, dissected and the soft tissues ...ere carefully separated from the hard shells. Both the soft tissue and the shells were placed separately in an oven (REMI) at 60°C overnight to determine their dry weights (40). Condition index (CI) was then calculated as follows:

$$\text{Condition Index} = \frac{\text{Dry soft tissue weight(g)}}{\text{Dry shell weight(g)}} X\ 100$$

Statistical Analyses

Statistical analyses of the data were carried out using IBM SPSS 23 statistical software package. The data were tested to meet the assumptions of normality and homogeneity prior to subsequent analyses by linear models. The data of the MN test and comet assay are expressed as percentage values and were therefore arc sine transformed whereas the data of CAT, AChE and MDA assays were log transformed. A one-way ANOVA was applied to test the effect of treatment on the % MNi, % Tail DNA, CAT, AChE, MDA and CI with a post hoc Dunnet's test to compare the different groups with the control within the same treatment group. Pearson's correlation with scatter plots were also used to test the relationship between the all the parameters. The data were considered to be statistically significant at $p < 0.05$.

Results

The dose response data of the MN test, comet assay, catalase assay, acetyl cholinesterase assay, malondialdehyde assay and condition index are presented in figure 1 (a-f). A significant dose dependent increase of DNA damage in the form of % MNi was observed in the gill cells of *M. casta* at all the concentrations of Cd ($p < 0.05$).

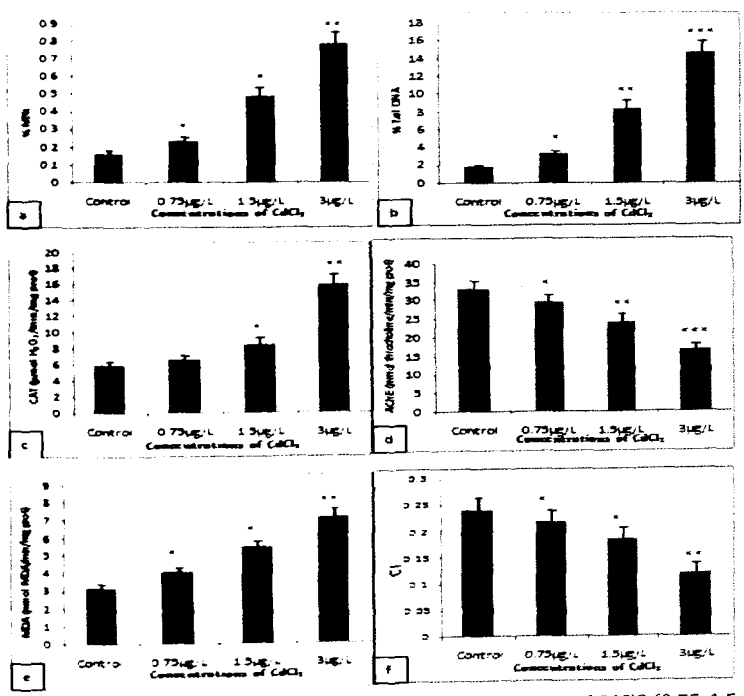

Figure 1: [Variations of biomarker responses (a-f) in M. casta exposed to different concentrations of CdCl2 (0.75, 1.5 and 3 µg/L; Number of replicates = 3). % MNi: Percentage Micronuclei, CAT: catalase, AChE: acetylcholinesterase, MDA: Malondialdehyde, CI: condition index. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

...A damage in the form of % tail DNA was also found to be ...significantly high at the 0.75 µg/L and 1.5 µg/L of Cd concentrations ...and was extremely significant at the 3µg/L concentration (p< ...001). The CAT activity did not change significantly at the 0.75 ...g/L concentration but increased significantly at the 1.5 µg/L (p ...0.05) and the 3 µg/L (p < 0.05) of Cd. AChE activity was found ...decrease in a dose-dependent manner whereas MDA activity ...showed an increasing trend with an increase in the concentration ...of Cd and was significant at all the doses (p < 0.05). The CI ratio ...also decreased significantly with an increase in the concentration ...of Cd (p < 0.05).

The effects of different concentrations of Cd on the ...variance of different tests are indicated in the one-way ANOVA (Table 1).

Table 1: one-way ANOVA of different concentrations of Cd on different biomarker responses in *M. casta*

Dependent variable	Independent variable (Treatment)	
	F value	p
% MNi	926.86	< 0.001 ***
% Tail DNA	1551.42	< 0.001 ***
CAT	535.95	< 0.001 ***
AChE	315.46	< 0.001 ***
MDA	515.75	< 0.001 ***
CI	124.21	< 0.001 ***

...between DNA damage and the CI ratio. The scatterplots (Figure ...2) further illustrate the associations between DNA damage and the other parameters.

A high positive correlation was observed between the % MNiand the % tail DNA (R = 0.95) as well as between both the genotoxicity parameters and the activities of CAT and MDA. However, a high negative correlation was observed between DNA damage and AChE levels (R = -0.93, -0.94) as well as Different concentrations of Cd effects contributed the most to the % Tail DNA (F = 1551.42, p < 0.001) followed by the % MNi (F = 926.86, p < 0.001). The activities of CAT, AChE and MDA and the condition index ratio were also significantly influenced by the different concentrations of Cd.

The correlation matrix indicating the association between the different parameters is given in (Table 2).

Table 2: Correlation matrix between the associations of the biomarker responses in *M. casta*

	% MNi	% Tail DNA	CAT	AChE	MDA	CI
% MNi	-					
%Tail DNA	0.95	-				
CAT	0.92	0.92	-			
AChE	-0.93	-0.94	-0.92	-		
MDA	0.95	0.96	0.89	-0.92	-	
CI	-0.86	-0.90	-0.87	0.87	-0.85	-

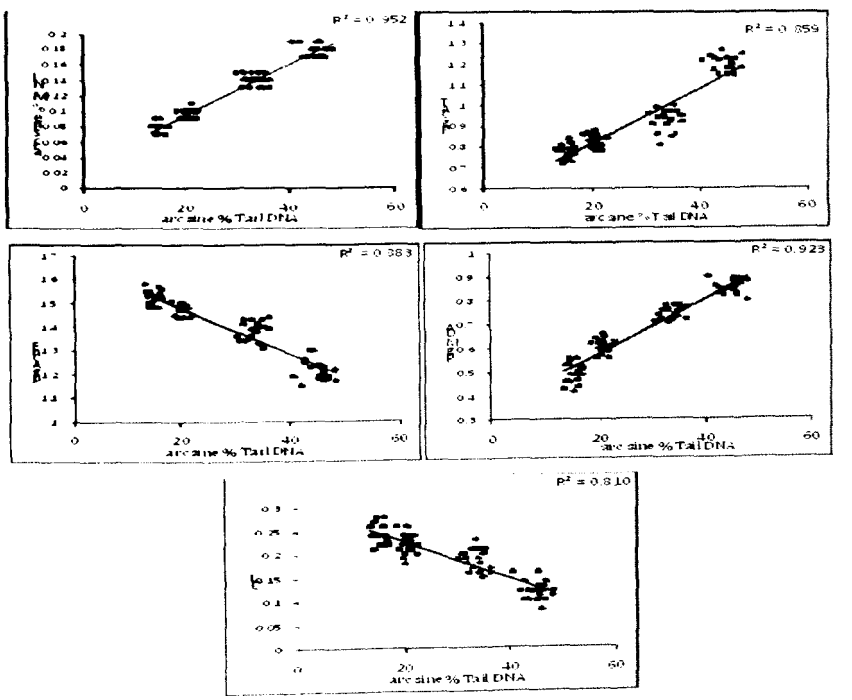

Figure 2: Scatter plots depicting the associations of DNA damage with other biomarker responses in *M. casta* exposed to different concentrations of CdCl2 (0.75, 1.5 and 3 µg/L; Number of replicates = 3). % MNi: Percentage Micronuclei, CAT: catalase, AChE: acetyl cholinesterase, MDA: Malondialdehyde, CI: condition index.

Discussion

The present study demonstrates the genotoxicity, neurotoxicity, oxidative stress and deteriorated condition induced by Cd in *M. casta* as indicated by the MN test, comet assay, AChE assay CAT assay, MDA assay and condition index. Cd was found to induce DNA damage in the gill cells of M. casta at all the concentrations studied. This is in agreement with the studies of Slobodskova et al. (41) where in they observed significant DNA damage induced by Cd in the gill cells of the clam, *Corbicula japonica*. Our observations were also on par with that of Sarkar et al. (31) in which they reported a significant increase of DNA damage with a concurrent decrease of DNA integrity in the gill cells of a marine gastropod, *Nerita chamaeleon* exposed to various concentrations of $CdCl_2$. In another study, Cd was found to be clastogenic in the Pacific oyster *(Crassostrea gigas)*, affecting the number of chromosomes in somatic cells significantly compared to control groups (42).

Significant increases in CAT activity were observed at the 1.5 µg/L and 3 µg/L concentrations. Similar observations were also reported by Macías-Mayorga et al. (43) in which *Crassostrea angulata* exposed to Cd showed an increase of CAT activity up to 7 days of exposure after which it was found to decrease significantly. Further, they attributed this oxidative stress in bivalves to the exposure to Cd. Liu et al. (44) proposed that Cd may generate free radicals by interfering with cellular antioxidant systems such as CAT.

Similarly, another consequence of oxidative stress was found to occur in *M. casta* as observed by the increase of a lipid peroxidation product, malondialdehyde (MDA). Dovzhenko et al. (45) also reported a similar increase of MDA in the bivalve *Modiolus modiolus* exposed to Cd. The increase in MDA and other lipid peroxidation products lead to a decrease in the total oxygen radical scavenging activity. As a result, there is an accumulation of Reactive Oxygen Species (ROS) leading to oxidative stress in the organism. These ROS in turn affect DNA causing modification of DNA bases and DNA strand breaks (46,47). Alternatively, Malondialdehyde (MDA) which is also highly mutagenic may form adducts with DNA and induce DNA damage (48). Our results are also supported by the observations of Xia et al. (30) in which an increase in the activities of both CAT and MDA in *M. meretrix* exposed to different concentrations of Cd, which in turn induced the apoptosis of hepatopancreatic cells. Dailianis et al. (49) suggested that Cd may induce the formation of ROS and DNA damage by stimulating the production of Protein Kinase C (PKC) via adrenergic receptors. Therefore, based on the strong correlation between DNA damage and oxidative stress parameters, the DNA damage observed in *M. casta* in the present study may be attributed to oxidative stress as a result of Cd exposure.

We also observed a significant positive correlation between the frequencies of % MNi and % tail DNA (R = 0.95) which are represented in the form of scatter plots (Figure 2). The comet assay is able to detect repairable DNA damage such as DNA strand breakages, whereas the MN test detects more persistent DNA damage that are more difficult to repair (50,51). These micronuclei are formed when a whole chromosome or a fragment of a chromosome does not get incorporated into either of the two daughter cells during cell division due to aneugenic agents that affect the spindle apparatus or clastogenic agents that damage and break the chromosome (52). Thus these two tests reflect different forms of environmental stress. The positive correlation in our study may be due to the conversion of the short term reversible damage to long term irreversible damage as a result of persistent Cd exposure.

Cd was also found to be neurotoxic in the bivalves as seen by the decreased concentration of AChE. The primary function of AChE is to catalyze the rapid hydrolysis of the neurotransmitter Acetylcholine (ACh) in the synaptic cleft thus terminating synaptic transmission. Cd may thus disrupt the function of AChE leading to an accumulation of ACh and overstimulation of cholinergic receptors. Our results are in agreement with that of Machreki-Ajmi and Hamza-Chaffai(53) in which cockles *(Cerastoderma glaucum)* transplanted from an unpolluted site to a site contaminated with Cd exhibited a significant inhibition of AChE activity. Our studies are also comparable with those of Dellali et al. (54) in which clams *(Ruditapes decussatus)* and mussels *(Mytilus galloprovincialis)* collected from sites polluted with heavy metals exhibited decreased acetylcholinesterase activity compared to those collected from unpolluted sites. Although the exact mechanism by which Cd causes inhibition of AChE in bivalves is not known, one possible mechanism may be due to ROS-mediated oxidative stress which is also seen to be negatively correlated in the present study (44).

A significant negative correlation was observed between the MN test and CI (R = -0.86) as well as between the comet assay and CI (R = 0.9). A similar negative correlation between condition index and tissue levels of environmental contaminants of *Littorina littorea, Mytilus edulis* and *Cerastoderma edule* in a river system (Milford Haven Waterway) of Wales, UK was reported by Langston et al. (55). This decrease of condition of the organism may be attributed to altered DNA function and thereby resulting in an altered protein function which is ultimately required for normal physiological processes. The physiological state of the bivalve can also lead to changes in its feeding activity thereby altering its life cycle as a consequence (56). Another reason for the decrease in the CI ratio could possibly be the survival adaptive response of *M. casta* wherein they reduce the filtration rate or closure of the shell on exposure to contaminants (57).

Conclusions

The present study revealed that Cd (0.75 µg/L, 1.5 µg/L and 3 µg/L) induced DNA damage in *M. casta* which was caused as a result of oxidative stress. Increasing concentrations of Cd also inhibited the activity of AChE and lowered the condition index ratio. The comet assay and the micronucleus test along with the biomarkers of oxidative stress such as CAT and MDA, AChE assay and condition index can be reliably used to assess the genotoxicity of Cd in *M. casta* in the environment. Hence, the regular monitoring of estuaries for contaminants such as Cd is

of utmost importance as the persistence of these contaminants could lead to significant decline in the natural populations of bivalves and may also pose a threat to the humans consuming them.

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Genotoxic Biomarkers as Indicators of Marine Pollution

17

M.K. Praveen Kumar, Avelyno D'costa, and S.K. Shyama

Abstract

The marine environment is the recipient of a wide range of pollutants including sewage, heavy metals, nanoparticles, petroleum hydrocarbons, and radiation which will ultimately affect the natural populations of aquatic organisms. These pollutants affect the integrity of the genome and lead to DNA damage. Genotoxicity tests such as the micronucleus test and comet assay are sensitive, reliable, and powerful techniques which are employed to assess the DNA damage. These tests can be designed on a wide variety of cells from any marine organism and can be performed either in the field or in the laboratory. The micronucleus test and comet assay can also be used as biomarkers along with other biochemical tests to evaluate the toxicity status of a particular marine water body. This review explores the use of these tests in different organisms under varying degrees of marine pollution. Further, it is recommended to use these two parameters together in order to confirm the holistic mutagenic/genotoxic effect of the test agents.

17.1 Introduction

Marine fauna, such as fish and bivalves, play a very crucial role in an aquatic ecosystem as consumers. Pollutants often get accumulated in sediment as well as the organisms of different trophic levels in aquatic ecosystems, including the benthic and pelagic animals. Fishes are the most predominant pelagic fauna in an aquatic environment representing the primary/secondary/tertiary consumers; whereas, bivalves are mostly sedentary in nature and are filter feeders. Larger fishes being positioned at the higher trophic level in our food chain can readily accumulate a variety of contaminants by ingestion of smaller species. Man, being at the apex of this food chain consumes varieties of fishes and bivalves as sea food and becomes the final recipient of these pollutants.

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Biomonitoring of water bodies employing its natural populations of flora/fauna is of utmost importance to understand its pollution status. Ample reports are available on environmental monitoring/biomonitoring which are routinely carried out to assess the genotoxicity status of probable polluted water bodies and are compared with reference/pristine sites (Osman 2014). Exposure of fishes to toxic substances may damage their genome or in other words, exhibit genotoxic effects. Genotoxicity induces DNA changes leading to various diseases including cancer, reduced reproductive competence, teratogenicity, and may be even fatal, resulting in low fish catch. Further this may affect the normal food chain and result in a deleterious shift in the biotic community of this coastal ecosystem. These will also result in a drastic reduction in the quality/palatability of sea food (Chen and White 2004). Sea food is a major route of exposure of human populations to toxic chemicals. Fish and shellfish have been recognized as major mediators for contaminant transfer to humans and cause of innumerable sufferings in man (Al-Sabati and Metcalfe 1995). Due to pollution of the coastal environment with genotoxic substances, relevant genotoxicity tests are quickly gaining significance and several techniques are being developed to detect DNA damage and to identify these pollutants (Osman 2014).

17.2 Environmental Pollution

Marine pollution includes a range of threats such as runoffs, oil spills, untreated sewage discharge, heavy siltation, persistent organic pollutants (POPs), heavy metals from mining by-products, radioactive substances, and dumping of garbage. Many of these pollutants get collected at various depths, where they are consumed by marine organisms and are introduced into the global food chain. These pollutants may then ultimately get accumulated in the sediment and can pose a reverse threat to the benthic fauna of the aquatic ecosystem (Chen and White 2004). Polycyclic aromatic hydrocarbons, polychlorinated biphenyls, and polybrominated diphenyl ethers, when taken up by the body, form active metabolites which then cause DNA adducts and subsequent DNA damage (Wang et al. 2008). Barsiene et al. (2006a) observed a high incidence of micronuclei in the hemocytes of flounder (*Platichthys flesus*) which were contaminated with high concentrations of organic pollutants, evident by the presence of PAH metabolites in the bile.

Heavy metals often get accumulated in the marine environment at concentrations below the normal permissible levels by inputs from various sources such as industrial and domestic wastes. Most of the metals get readily concentrated and accumulated by aquatic organisms are relatively toxic even at fairly low concentrations. Discharge of heavy metals into river or any marine environment can change both marine species diversity and ecosystems, due to their toxicity and accumulative behavior (Bakan and Böke Özkoç 2007; Bat et al. 2009). Marine organisms such as fish tend to accumulate heavy metals many times higher than the concentration of that in water or sediment (Bat et al. 2009).

The uptake of xenobiotic contaminants, such as PCBs and PAHs, is highly detrimental to the biological integrity as well as the physiological functions of marine organisms. Many of these pollutants are chemical carcinogens and mutagens with the capacity to cause various types of DNA damage. Benzo[α]pyrene, a representative PAH, is reported to be converted at cellular level to the ROS, diol-epoxide (BaPDE), which can form stable adduct with DNA resulting into DNA strand breaks (Pisoni et al. 2004; Bihari and Fafandel 2004).

Nanogenotoxicology, which deals with the studies on the genotoxic effects of nanomaterials and nanodevices, is emerging as an important discipline along with the rapid expansion of the nanotechnology industry. Nanoparticles (NPs) are being used as a part of everyday products: thus, it is necessary to know and assess the possible toxic effects that are associated with them. The extremely small size of nanomaterials also means that they much more readily gain entry into the human body than larger-sized particles. Particles may gain access to the body by inhalation, ingestion, or through dermal contact, either deliberately or accidentally. Many metal NPs induce DNA strand breaks, oxidative DNA damage, mutations, and chromosomal aberrations. Particulate heavy metals (nano-sized) may inter-

act with fish or other organisms as follows: (1) adsorption to the surface, i.e., cell, organ, or whole body, (2) cellular internalization, (3) dissolution of ions from the NP, and (4) mechanistic nano-effects such as formation of reactive oxygen species (ROS) (Baker et al. 2014). Gomes et al. (2013) exposed mussels to 10 μg/L of copper oxide (CuO) and silver (Ag) NPs for 15 days and reported DNA damage in hemocytes in the form of DNA strand breaks by the comet assay.

Radiations of various kinds widely used across the globe for diverse purposes are one of the major pollutants of our water bodies. Organisms get exposed to a wide range of radiation from these sources including radioactive effluents. Water bodies may get contaminated from industrial effluents intentionally or from nuclear facilities by accident. With most of the nuclear reactors being located in and around coastal areas, seas are often the ultimate recipient of radioactive contaminants (Praveen Kumar et al. 2014). Besides this, the nuclear accidents such as Chernobyl nuclear accident or the recent Fukushima disaster are the wake-up calls for employing radiation protection/safety of humans and the environment (Dallas et al. 2012). Since the risks of ionizing radiation to nonhuman biota being of considerable current interest to both the International Commission on Radiological Protection (ICRP) and the International Atomic Energy Agency (IAEA), they strongly recommend the impact assessment of radiation on those organisms (IAEA 1992; ICRP 2007). The major objective of the two international agencies, viz., FASSET (Framework for Assessment of Environmental Impact) and EPIC (Environmental Protection from Ionizing Contaminants in the Arctic), is to develop a methodology for protecting nonhuman organisms from ionizing contaminants (EPIC Project 2001; FASSET Project 2001).

17.3 Uses of Genotoxicity Parameters in Evaluation of Marine Pollution

The genotoxic effects of physical and chemical agents/pollutants can be monitored using a broad range of both in vivo and in vitro biomarker assays. The use of "biomarkers" in marine species has become a major tool for biomonitoring and assessing the environmental quality. This is based on the responses at the molecular and cellular level which represent the earliest signals of environmental disturbance (Depledge 1994; Serafim et al. 2011). Cytogenetic endpoints are proven as sensitive early biomarkers (Dallas and Jha 2015). Micronucleus (MN) test and comet assays are well-known genotoxicity tests often employed to predict mutagenicity/carcinogenicity of chemicals, including pharmaceuticals under in vivo and in vitro conditions (Shyama et al. 1991; Tice et al. 2000; Kirkland et al. 2005; Anbumani and Mohankumar 2012; Kadam et al. 2013; Dallas et al. 2013; Dasgupta et al. 2014; Guilherme et al. 2014; Pandey et al. 2014; Osman 2014; Praveen Kumar et al. 2015). These parameters are routinely used as biomarkers for monitoring marine pollution by genotoxic contaminants and can be combined with other physiological and biochemical biomarkers to fully assess the pollution status of various water bodies (Bolognesi and Cirillo 2014).

17.4 Micronucleus Test

Micronucleus test is an easy and useful test for the in vitro and in vivo genotoxicity assessment in marine organisms. Micronuclei result from acentric chromosome fragments or whole chromosomes lagging behind during metaphase to anaphase transition induced by clastogens or by spindle dysfunctions, respectively. Micronucleus test is routinely used for assessing the genotoxicity of compounds released from industries into aquatic environments (Barsiene et al. 2004; Cavaş and Ergene-Gozukara 2005a, b; Da Silva Souza and Fontanetti 2006). Fishes and bivalves are generally used to screen the potential genotoxicity of polluted water bodies (Bolognesi and Hayashi 2011). Natural populations of fishes collected from two different radiation-polluted sites showed high levels of MN (Ilyinskikh et al. 1998). Increased frequency of micronuclei was also reported in three fish species (*Platichthys flesus*, *Clupea harengus*, and *Zoarces viviparus*) collected from different regions of the Baltic Sea

over a period of 3 years suggesting the periodical increase of pollution in the study site (Barsiene et al. 2012). X rays induced significant number of micronuclei in the erythrocytes of *Cyprinus carpio* (Gustavino et al. 2001) and also in the gill cells of *Oryzias latipes* (Takai et al. 2004a, b). Low-dose gamma radiation induced significant increase of micronuclei in fish cell lines, in vitro (Cassidy et al. 2007). A significant number of micronuclei were observed in the erythrocytes of the gamma-irradiated (5 Gy) fish, *Catla catla* (Hamilton) (Anbumani and Mohankumar 2011, 2012). An increase in the frequency of micronuclei in caged mussels (*Mytilus galloprovincialis*) transplanted at an offshore oil platform in the Adriatic Sea (Gorbi et al. 2008) supports the potential of these organisms to be used as biomonitoring species. Tritiated water (HTO) and tritiated glycine (T-Gly) induced a significant number of micronuclei in the hemocytes of *Mytilus edulis* (Jaeschke et al. 2010). Petroleum hydrocarbons of oil spills too induce genotoxicity as evident by the formation of micronuclei in the erythrocytes of flounder (*Platichthys flesus*) and gills of blue mussel (*Mytilus edulis*) (Barsiene et al. 2006b). Sacchi et al. (2013) reported the induction of micronuclei in the bivalve *Ruditapes philippinarum* at an estuarine coastal site polluted with polycyclic aromatic hydrocarbons and trace metals. MN levels in mussels (*Mytilus galloprovincialis*) collected from 17 sites along the Spanish Mediterranean coast were found to be the highest from metal-polluted sites (Fernandez et al. 2011). They also reported that environmental factors such as water temperature were able to influence the levels of metal-induced MN. Fishes collected from the Paraguay river within the Pantanal, Brazil, which is the world's largest wetland area and a region rich in biodiversity, revealed significantly high frequencies of MN in the erythrocytes probably due to the discharge of tannery effluents (Pimenta et al. 2013).

17.5 Comet Assay

The comet assay or single-cell gel electrophoresis (SCGE) is a rapid and sensitive technique that detects DNA single- or double-strand breaks and measures the level of migration of DNA from individual cell nuclei. The amount of the DNA in the tail region (tail DNA) of comet assay is commonly used for quantifying DNA strand breakage and represents the most reliable parameter (Mitchelmore and Chipman 1998). This test is also widely used for in vivo assessment in marine organisms (Fernández-Tajes et al. 2011; Frenzilli and Lyons 2013). Mussels are globally used as bioindicators for pollution of coastal and estuarine environments by metals and radionuclides (Lonsdale et al. 2009). Hagger et al. (2005) have reported the β- radiation induced genotoxic effects of tritiated water in the early life stages of the marine mollusk, *Mytilus edulis*. Further, the genotoxic effects of tritium (^{3}H) in the adult life stage of *Mytilus edulis* have been evaluated employing comet assay (indicating DNA single-strand breaks/alkali labile sites) in the hemocytes of radiation-exposed individuals (Jha et al. 2005, 2006). External and internal dose rates of ionizing radiation altered the DNA strand breakage in marine mussel *Mytilus edulis* (Alamri et al. 2012).

Polycyclic aromatic hydrocarbons were found to induce DNA strand breaks in the hemocytes and gill cells of three bivalve species (*Venerupis pullastra*, *Cerastoderma edule*, and *Mytilus galloprovincialis*) collected from an estuary contaminated with petroleum products (Fernandez-Tejes et al. 2011). Bottom feeders such as soles (*Solea senegalensis*) exhibited increased DNA strand breaks when they were exposed to sediment contaminated with heavy metals and PAHs and PCBs (Costa et al. 2008). Snails (*Littorina littorea*) and mussels (*Mytilus edulis*) collected from a contaminated harbor showed high levels of DNA damage due to presence of heavy metals and butyltin compounds (Rank 2009). Binelli et al. (2007) correlated the increase in DNA strand breaks in soft tissues of zebra mussels (*Dreissena polymorpha*) with chemicals such as polycyclic aromatic hydrocarbons, polybrominated diphenyl ethers, Dichlorodiphenyltrichloroethanes (DDTs), and hexachlorobenzene in the Lake Maggiore, Italy.

The most severe DNA lesions induced by ionizing toxicants are single- and double-strand breaks (Collins 2004). The alkaline single-cell gel electrophoresis assay allows the early detec-

tion of single-strand DNA breaks which may be induced by a range of genotoxic agents including radiation (Mayalpa et al. 1998; Kadam et al. 2013). Gamma irradiation is reported to induce DNA damage and cell cycle perturbations in the fish, *Catla catla* (Anbumani et al. 2012; Anbumani and Mohankumar 2015). A dose-dependent DNA damage was observed in zebrafsh exposed to ionizing radiation (Knowles 2002; Jarvis and Knowles 2003; Lemos et al. 2014). An increase in % tail DNA was observed in caged as well as feral European chubs (*Leuciscus cephalus*) from rivers polluted with a combination of PAHs, PCBs, organochlorine pesticides (OCPs), and heavy metals around Birmingham, UK (Winter et al. 2004). De Andrade et al. (2004) also reported increased DNA damage in mullet and sea catfish from two rivers in Brazil that were possibly contaminated with heavy metals and hydrocarbons. Dabs (*Limanda limanda*) collected from the mouth of the Seine estuary in France exhibited high DNA damage (% tail DNA) and were found to be positively correlated with the concentration of biliary PAH metabolites (Devier et al. 2013). Cockles (*Cerastoderma edule*) and blue mussels (*Mytilus edulis*) collected from an estuary in South West England exhibited high DNA damage due to the presence of heavy metals discharged by industries (Dallas et al. 2012). Almeida et al. (2013) reported low levels of DNA damage from a coastal lagoon in Portugal and has also demonstrated the use of the comet assay to evaluate genotoxicity from places of low contamination.

17.6 Conclusions

The presence of large quantities of pollutants such as heavy metals, petroleum hydrocarbons, radiation, and nanoparticles causes several adverse effects in marine fauna and may affect the fish catch and palatability. Further, these pollutants may enter the food chain and ultimately affect humans. The micronucleus test detects chromosomal and genomic mutations (chromosomal damage and/or alteration of mitotic spindles), whereas the alkaline comet assay detects primary DNA damage, expressed as single-strand breaks, including the ones which are associated with incompletely repaired excision. A combined use of MN test and alkaline single-cell gel electrophoresis confirms the possible single as well as double-strand breaks induced by genotoxic agents. Accordingly, it is recommended to use these two parameters together in order to confirm the holistic mutagenic/genotoxic effect of the test agents (Figs. 17.1 and 17.2).

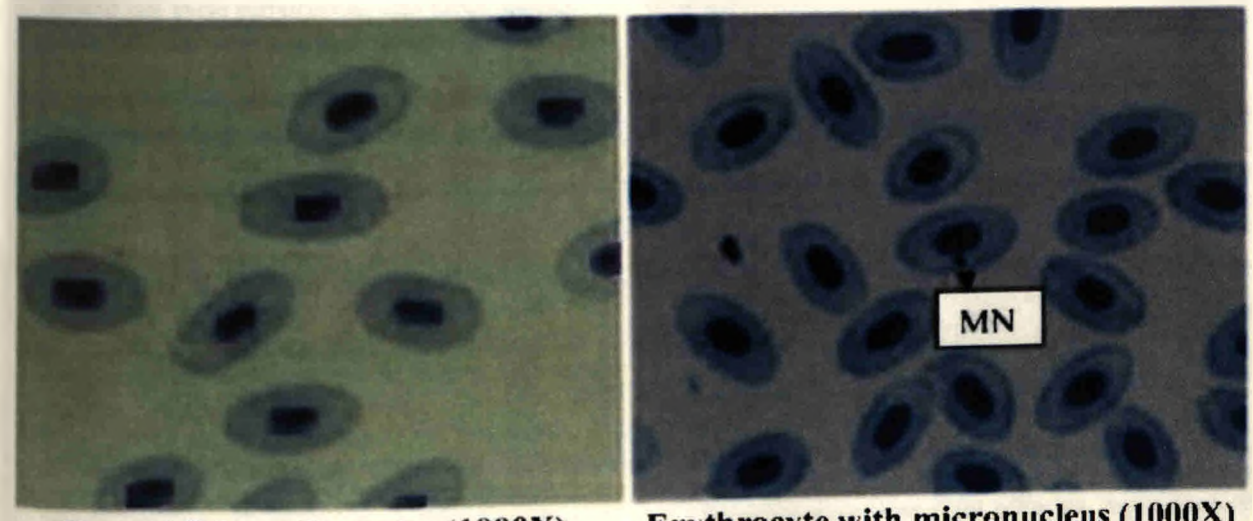

Fig. 17.1 Normal erythrocytes and erythrocyte with a micronucleus in zebra fish

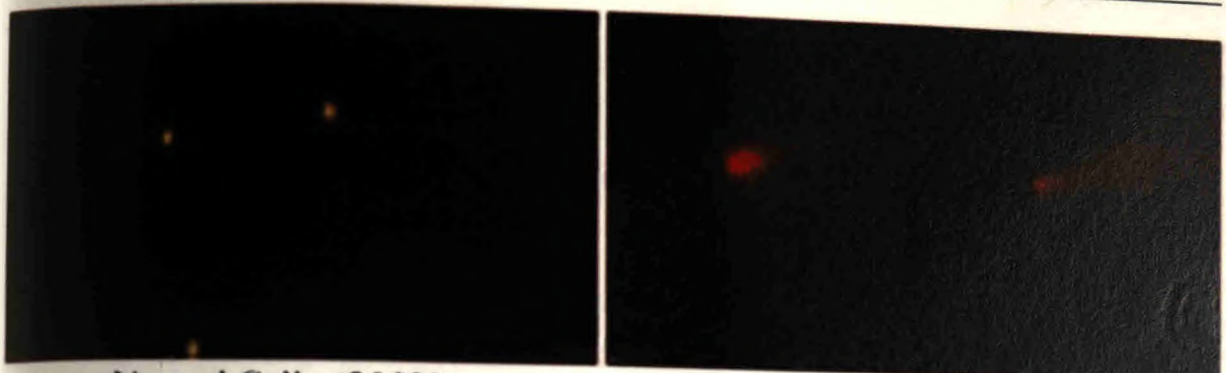

Fig.17.2 Comet assay in erythrocytes of zebra fish

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