Ecotoxicological Studies of Heavy Metals on Edible Oysters (*Crassostrea* spp.) from Goa Coast, India

A Thesis submitted to Goa University for the Award of the Degree of

DOCTOR OF PHILOSOPHY

in

MARINE SCIENCES

By

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GOA UNIVERSITY
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India

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April 2018

CERTIFICATE

This is to certify that the thesis entitled "Ecotoxicological studies of heavy metals on edible oyster *Crassostrea* spp. from Goa coast, India", submitted by Ms. Prachi Shankar Shenai-Tirodkar, for the award of the degree of Doctor of Philosophy in Marine Sciences is based on original studies carried out by her under my supervision.

The thesis or any part therefore has not been previously submitted for any degree or diploma in any universities or institutions.

Place: Dona Paula

Dr. Mangesh U. Gauns
Research Guide
Principal Scientist
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Place: Dona Paula

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STATEMENT

As required under the University Ordinance OB 9.9, I state that the present thesis entitled "Ecotoxicological studies of heavy metals on edible oyster *Crassostrea* spp. from Goa coast, India" is my original contribution and the same has not been submitted on any previous occasion. To the best of my knowledge, the present study is the first comprehensive work of its kind from the area mentioned.

The literature related to the problems investigated has been appropriately cited. Due acknowledgements have been made wherever facilities and suggestions have been availed of.

Place: Goa, India.

Prachi Shankar Shenai-Tirodkar

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Prachi Shankar Shenai-Tirodkar

Dedicated to

My Parents and Husband

Whose love and affections stands before me as a constant source of inspiration in my life

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List of Abbreviations

°C	Degree Celsius
µg/l	Microgram per liter
µm	Micrometer
µg C/l	Microgram Carbon per liter
µmol/l	Micromole per liter
$^{\bullet}OH$	Hydroxyl radical
AE	Assimilation Efficiency
ANOVA	Analysis of Variance
APDC	Ammonium Pyrolidine Dithiocarbamate
ASP	Aspartate
ATP	Adenosine Triphosphate
BHT	Butylated Hydroxytoluene
CA	Cluster Analysis
CAT	Catalase
CB	Chicalim Bay
Cd	Cadmium
cDNA	Complementary DNA
CDNB	2,4-Dinitrochlorobenzene
ChB	Chapora Bay
Chl *a*	Chlorophyll *a*
cm	Centimeter
CSIR	Council of Scientific and Industrial Research
Cu	Copper
$CuSO_4$	Copper sulfate
DCA	Detrended Correspondence Analysis
DNA	Deoxyribonucleic acid
DO	Dissolved Oxygen
DORM - 4	Fish protein certified reference material for trace metal

DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ERL	Effect Range Low
ETC	Electron Transport Chain
ETM	Estuarine Turbidity Maximum
EU	European Union
FAO	Food and Agricultural Organization
Fe	Iron
Flame - AAS	Flame Atomic Absorption Spectrometry
g	Gram
g/cm	Gram per centimeter
GF-AAS	Graphite Furnace Atomic Absorption Spectrometry
GPX	Glutathione Peroxidase
GR	Glutathione Reductase
GSH	Reduced Glutathione
GST	Glutathione-S-Transferase
H_2O_2	Hydrogen peroxide
HCL	Hydrochloric acid
$HCLO_4$	Perchloric acid
HF	Hydrogen Fluoride
Hg	Mercury
His	Histidine
HNO_3	Nitric acid
hrs	Hours
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
ICP-OES	Inductively Coupled Plasma Optical Emission Spectrometry
KCL	Potassium chloride
KDa	Kilodalton
Kg/m/yr	Kilogram per meter per year
km	Kilometer
$KNaC_4H_4O_6$	Potassium sodium tartrate

LPO	Lipid Peroxidation
m	Meter
MAG -1	Marine Sediment Certified Reference Material
MANOVA	Multivariate Analysis of Variance
mg/kg	Milligram per kilogram
MIBK	Methyl Isobutyl Ketone
mM	Millimolar
mm	Millimeter
Mn	Manganese
Mon	Monsoon
mRNA	Messenger RNA
MT	Metallothionein
Na_2CO_3	Sodium carbonate
NaOH	Sodium hydroxide
NC	Nerul Creek
Ni	Nickel
NIO	National Institute of Oceanography
NO_2	Nitrite
NO_3	Nitrate
O_2	Oxygen
$O_2^{\bullet-}$	Superoxide radical
OC	Organic Carbon
Pb	Lead
$Pb(NO_3)_2$	Lead nitrate
PBS	Phosphate Buffered Saline
PCA	Principle Component Analysis
PERMANOVA	Permutational Multivariate Analysis of Variance
Phaeo.	Phaeopigment
PO_4	Phosphate
POC	Particulate Organic Carbon
PostM	Post-monsoon

PreM1	Pre-monsoon 1
PreM2	Pre-monsoon 2
PUFA	Polyunsaturated Fatty Acids
RDA	Redundancy Analysis
$RO^{\bullet}$	Alcoxyl radical
$ROO^{\bullet}$	Hydroperoxyl radical
ROS	Reactive Oxygen Species
RSD	Relative Standard Deviation
SD	Standard Deviation
SDS	Sodium Dodecyl Sulfate
-SH	Sulfhydryl group
SOD	Superoxide Dismutase
sp.	Species
SPM	Suspended Particulate Matter
TBA	Thiobarbituric acid
TBARS	Thiobarbituric Acid Reactive Substances
TC	Total Carbon
TCA	Trichloroacetic Acid
TN	Total Nitrogen
TOC	Total Organic Carbon
TSS	Total Suspended Solids
USFDA	United States Food and Drug Administration
WHO	World Health Organization

Chapter 1

General Introduction

Chapter 1

1.1 Oyster

The oyster refers to bivalve molluscs with rough irregular shells that live in marine or brackish water. The word oyster originated from French word *oistre* via Latin from Greek word ostreon. Oysters inhabit coastal and estuarine regions from temperate to tropical latitudes (64° N and 44° S) except for the Artic and Antartic regions (Galtsoff, 1964; Ruesink et al., 2005). With few exceptions oysters grow well in shallow water, their vertical distribution extending from a level approximately halfway between high and low tide levels to the sublittoral zone (upto 100 feet) (Galtsoff, 1964). Edible oyster mainly belongs to genera *Ostrea*, *Crassostrea*, and *Saccostrea*. The classification of oysters is based on the form and structure of larval shell, mode of reproduction, life history, adult shell morphology, and foot shape.

1.2 Genus *Crassostrea* (Sacco, 1897)

Classification:

Kingdom: Animalia

Phylum: Mollusca

Class: Bivalvia

Order: Ostreoida

Family: Ostreidae

Genus: *Crassostrea*

The description of the Genus *Crassostrea* was given by Galtsoff (1964). Usually shell of *Crassostrea* is elongated and externally rough in texture. The lower (left) valve is deep,

cup shaped, and recessed under hinge, and cemented to the hard substratum. The upper (right) valve is opercular, and flat except sometimes upraised at the rim. The hinge is toothless with linear margin, adductor muscle scar present dorso-laterally and towards the lip. The mantle or pallium is fleshy fold of tissue that covers the internal organs. The gill, ostia and eggs are small in size. Pair of gills comprised of an inner and outer demibranch and rectum does not transverse through ventricle. The promyal chamber is large in size present on the right side of the body. They are non-incubatory or oviparous species.

1.2.1 Habitat

Crassostrea sp. is euryhaline and occurs at intertidal and subtidal level in creeks, bays, estuaries, backwaters, and invades upto 100 feet, coastal shallows and inshore zone. They attach themselves to a hard substratum such as rocks, stones, dead shells, corals, concrete cements, shipwrecks and covers extensive areas in coastal and estuarine regions. The intertidal/subtidal biogenic structures formed due to large aggregation of oysters and building of a habitat with significant surface complexity is known as oyster beds, oyster bottoms, oyster bars, oyster banks, or oyster reefs (Galtsoff, 1964; Bahr and Lanier, 1981; ASMFC, 2007).

1.3 Study Species

Two oyster species (*Crassostrea madrasensis* and *C. gryphoides*) were selected for the present study. Both the species occur in same habitat and show close resemblance in external shell morphology (Siddique and Ahmed, 2002) creating confusion among researchers during species identification (Plate 1.1). To overcome this problem, Durve

(1974) distinguished the two-species based on conchological and malacological characters. General description and their general distribution along the Indian coast is given below.

1.3.1 Crassostrea madrasensis (Preston, 1916)

Description: *Crassostrea madrasensis* inhabits the intertidal region from midlittoral zone to a depth of 17 m. It grows up to a length of 22 cm (shell length). Left shell valve is deep while the right one is slightly concave. Adductor muscle is elliptical or oblong kidney-shaped and dark purple in colour (Plate 1.2). Mantle is without or with slight pigmentation. If pigmentation is present, it is mainly on the middle and inner lobes. Anal portion of the rectum is 0.5 to 1 mm in length and slightly directed outwards. Anal opening is situated at the middle of the ventral margin of adductor muscle (Durve, 1974).

Distribution along the Indian coast: This species occurs along the east and west coast of India. In particular, it is found in coastal waters of Goa, Karnataka, Kerala, Tamil Nadu, Andhra Pradesh, Orissa and Andaman and Nicobar Islands (Dholakia, 2004).

1.3.2 Crassostrea gryphoides (von Schlotheim, 1813)

Description: *Crassostrea gryphoides* inhabits marine waters up to a depth of 7 m. It grows up to a length of 17 cm (shell length). Striations on adductor muscle scar are absent or obscure. Adductor muscles are roundish, bean-shaped, rarely oblong and elliptical. Mantle is pigmented and black in colour (Plate 1.3). Pigmentation of the mantle pronounced on the edge of the outer and inner lobes. The pigmentation also extends on the surface of the inner lobe. Anal portion of the rectum is 1 to 2 cm in length and

markedly directed outwards. The anal tip is simple, at times slightly funnel shaped. Anal opening situated at the corner of the posterior and ventral margins of the adductor muscle (Durve, 1974).

***Distribution along the Indian coast*:** This species is commonly found along the coastal waters of Gujarat, Maharashtra, Goa, Karnataka and West Bengal (Dholakia, 2004).

1.4 Biology of Oyster

1.4.1 Morphology and Anatomy

Morphology and anatomy of an oyster has been shown in Plate 1.4. The body of oyster is fully covered with two rough shells which hinged together present at the anterior end. However, variations in the shape of the oyster shells are expected within the same species from same area due to influence of overcrowding, orientation and substratum. This process is called xenomorphism in which shape is determined by contour of the substrate where they grow (Quayle and Newkirk, 1989). Shell is made up of three layers, all produced by the mantle. The outermost layer is a thin proteinaceous covering called the periostracum (peri=around; ostracum=shell). The middle layer is the prismatic layer composed of calcium carbonate. The innermost layer is the nacreous layer. Umbo is the oldest part of the shell which formed first, located at the hinge. Many factors such as temperature, salinity, food, stress, and disease affect the oyster's ability to produce shell (Durve and Bal, 1962). The internal organs of the oyster are covered and protected by the tegument known as a mantle or pallium which is also involved in the shell calcification (Eble and Scro, 1996). A pallial cavity is present between the two lobes of the mantle, and this free space further divided by the gills into inhalant and exhalant part where

seawater circulation occurs (there are no siphons). The adductor muscle, a prominent organ situated in the posterior region of the body, consists of an anterior translucent larger part and a smaller, white, crescent-shaped region. The adductor muscle functions to close the shell. Relaxation of the adductor muscle allows the valves to gape because of the resiliency of the hinge ligament (Eble and Scro, 1996). An oyster has a pair of gills, comprised of an inner and outer demibranch, present between the mantle and internal organs. In addition to the respiration function, gills also filter the edible particulate matter for food (Newell and Langdon, 1996). The mouth is surrounded by the labial palps which is located near hinge while anus is just occurres above the adductor muscle.

1.4.2 Reproduction and Growth

Oysters are protandrous hermaphrodite, where male predominate in smaller size classes and female favors in larger oyster (Asif, 1979; Durve, 1965). At the age of three, oysters reach their prime reproductive phase but they are entirely dependent on their environmental conditions for their reproduction and growth (Durve, 1965). The life-cycle of oyster is separated into two distinct stages, the planktonic pre-settlement – larval period and post-settlement juvenile – adult period (Figure 1.1). Under natural conditions, sexually matured oysters release their gametes (sperms and eggs) into the surrounding water column. Oyster requires an environmental signal such as optimum water temperature, salinity and amount of phytoplankton biomass for their spawning process. For example, after monsoon, increase in salinity stimulates the breeding in *C. madrasensis* which occurs continuously in marine but discontinuously in estuarine environment (Rao, 1951). Two spawning peaks for *C. madrasensis* have been reported:

first major peak occurs in October (post-monsoon) and second minor peak arises in June (monsoon) (Alam and Das, 1999). These spawning recruitment phase occurs between September and November have also been reported (Nurul Amin et al., 2008). Furthermore, *C. gryphoides* requires optimum salinity of 22.3 for egg development (Rao, 1951); whereas 13.2 and 28.6 for spawning, and maturation proceeds during winter period. The number of sex cells produced by oyster during one reproductive period varies, depending on the conditions of the environment. A total of 50-100 million eggs are produced by *C. madrasensis* (Biswas, 2004). It only takes one oyster to release its gonads to encourage other oysters to commence their spawning. Once the other oysters detect sperm in the water, they begin to release their own gonads (Biswas, 2004). Next, fertilization of eggs occurs usually in the surrounding water. After the oyster egg has been fertilized, egg drift with tides, waves and eddy currents and distribute in an uneven manner which further undergoes in cell division. The first stage of development occurs within few hours of fertilization. The gastrula stage is reached within 5-6 hours after fertilization where larva start swimming upwards. The developed trochophore larvae have cilia at one end which make them motile. Although they can move horizontally (to some extent vertically) but, they are still planktonic and subject to the whims of the currents. After fertilization, at the end of 20 hours the next larval stage called veliger larva is reached which has “D” shape structure. The veliger larva stage has complete digestive systems and with the help of velum larvae collects food particles. The larval oyster now resemble pediveligar stage on 13th day (Sawant, 1997) and is sometimes referred as eyed larva because of the presence of an eye spot which start moving from the water column to the bottom of the aquatic system. At this point larva settles to the bottom

and search for a suitable substrate to attach themselves. To find a suitable site, the larva crawls along the material with their foot to search the favorable substratum for its attachment (Murthy et al., 1999). After this, larva enters in a fixed stage of life-cycle and lies on their left (cupped) shell and holds on with the tip of foot. The byssus gland is located at the tip of the foot and is responsible for secreting the cement that hold the oysters permanently to the substrate. The material to which larva attaches is called cultch.

After adhesion, larva undergoes complete metamorphosis of internal anatomy and becomes a spat (Tibile and Singh, 2003). The foot and eye spot are adsorbed because the oyster never need them again. Within about 3 months the spat reach the size of a dime. Shell formation and growth are now the main concerns of the spat which depends on the several environmental factors. Temperature, salinity, pH, food availability, light are important factors that affect the physiology, stages of development, growth and distribution of oysters (Shumway, 1996; Sasikumar et al., 2007).

1.5 Feeding Behavior

Oyster is a filter feeder, generally extracting phytoplankton and other suspended particulate material (organic and inorganic) from the seawater. It can collect particles by size (<50 µm) at the level of the gills and labial palps (Bougrier et al., 1995; Barille et al., 1997, 2000). In particular, oyster has a capacity to select inanimate food and phytoplanktonic organisms such as diatoms, flagellates, nanoplanktonic spore and particulate matter of the algae at the level of gills and labial palps. Some common genera of flagellates and diatoms that oyster prefers include *Isochrysis* sp., *Monochrysis* sp.,

Dunaliela sp., *Chaetoceros* sp. and *Phaeodactylum* sp. But the food intake varies with the species, stage of growth, its pumping rate and environmental conditions and locations. Oyster also absorb dissolved organic matter (e.g. amino acids) from seawater via surface of the gill, palp and mantle (Guo et al., 2001; Hedouin et al., 2010). In tropical regions, in relatively sheltered area an oyster can pump for nearly 20 hours in a day (Dholakia, 2004). This feature facilitates oyster to grow fast. Oyster stores the excess of energy within its body in the form of glycogen and uses during the periods in which the oyster keep its shell closed in response to environmental changes.

1.6 Commercial Importance of *Crassostrea*

The genus *Crassostrea* includes several commercially exploited species with high demand as seafood all over the world. Oyster has largest production through aquaculture where several species are cultured in many parts of the world (FAO, 2016). Global capture of oyster in year 2014 were recorded 0.13 million tonnes (FAO, 2014). The commonly farmed commercial species includes pacific cupped oyster (*C. gigas*), American cupped oyster or Eastern oyster (*C. virginica*), European flat oyster or belon oyster (*Ostrea edulis*), Portuguese cupped oyster (*C. angulata*) and Sydney rock oyster (*Saccostrea glomerata*). Amongst, the major oyster producing countries are China, Japan, Korea, Mexico, France and the USA. On global scale, most popular and heavily harvested species are *C. virginica,* found in Atlantic waters from Canada to Argentina, and the *C. gigas*, found from Japan to Washington state and as far south as Australia. The modes of growth and population structures of *Crassostrea* giving them a supreme

importance in oyster fishery which contributes to the national economy of many countries including India (Vakily, 1992).

In India, the bivalve fishery is constituted mainly by clams (*Paphia malabarica*, *Meretrix casta*, *M. meretrix*, *Villorita cyprinoides*), mussel (*Perna viridis*), and oysters (*C. madrasensis*, *C. gryphoides*, *S. cucullata*) (Jones, 1970; Jones and Alagarswami, 1973). Thirteen species of oysters are commercially exploited from Indian waters: edible oysters (consist of 6 species), pearl oysters (6 species) and window-pane oyster (1 species) (Venkataraman and Wafar, 2005). James (1992) reported the presence of following six species of oysters in Indian waters: 1) Indian back water oyster, *C. madrasensis*, 2) Chinese oyster, *C. rivularis*, 3) West coast oyster, *C. gryphoides*, 4) Indian rock oyster, *S. cucullata*, 5) Bombay oyster, *Saxostrea cucullata*, 6) Giant oyster, *Hyostissa hyotis*. Among these, *C. madrasensis* and *C. gryphoides* are commercially important, widely distributed and abundantly found species along the Indian coast (Rao, 1974). Globally, oysters are consumed as a nutritional food due to presence of high content of protein, calcium, iron, minerals and vitamins (A, D, E, B1, B2, B6, B12, C) and is also rich in selenium and polyunsaturated fatty acids (PUFA) (Asha et al., 2014). *Crassostrea gryphoides* has shown potential as a cultivable species as it has an ability to attain the marketable size (4 cm) in relatively less time (0.7 cm/month) (Parulekar et al., 1984; Chatterji et al., 1985; Sawant, 1997). Furthermore, in many countries oyster shells are used in production of cement, calcium carbonate, sand lime bricks, quicklime (calcium oxide) and making of jewelry (Dholakia, 2004). Pearl oyster powder is used in manufacturing of medicine and cosmetics, and shell powder (a rich source of calcium)

serves as a diet supplement in feeding livestock and poultry. Recently, Harzhauser et al. (2016) named *Crassostrea* mainly *C. gryphoides* as a carbonate factory as its dense colonies has a potential to produce carbonate ~15 kAg/m/yr. In addition to Indian waters, *C. madrasensis* are commercially utilized in many parts of Asia (Angell, 1986) including Pakistan, Sri Lanka, Indonesia and Bangladesh waters (Indrasena and Wanninayake, 1994; Alam and Das, 1999; Ghazala and Muzammil, 2001; Siddique and Ahmed, 2002; Nurul Amin et al., 2008).

1.7 Significance of Oyster in Ecosystem

The benthic fauna is an important component in the trophic structure of the ecological pyramid, and exhibit wide variations in their abundance. Oysters typically belong to a group called benthos. Oyster reefs play numerous important ecological roles in world's marine ecosysytems. Particularly, in estuarine and coastal waters they provide important habitat for several species. In addition, they help in improvement of water circulation pattern and benthic zone stabilization. Oyster beds also play an important role in the cycling of nutrients within marine system specifically in translocation, transformation and remineralization of essential nutrients (Dame, 1996). Furthermore, being ecosystem engineers, they can modify the physical and chemical environment, with major contribution in estuarine populations, communities, and food webs (Jackson et al., 2001; Ruesink et al., 2006). Oyster reefs are valuable habitat which support large number of diverse communities, such as sponges, hydroids, bryozoans, barnacles, mussels, limpets, clams etc. In a detailed study, Wells (1961) found that at a time more than 300 marine invertebrate species may occupy an oyster reef.

However, oyster populations in many areas have declined or disappeared where they were once abundant. For example, Beck et al. (2011) estimated the loss of 85 % of oyster beds globally and that most of remaining natural oyster populations are in poor condition due to overfishing. Moreover, other factors such as habitat degradation due to coastal development and dredging, exposure to diseases, pollution, ocean acidification and sedimentation have contributed in varying degrees to estuarine and regional-scale declines in oyster populations.

1.8 Heavy Metal Pollution in Marine Environment

The term "heavy metal" is having no standard definition, but metals with a specific density greater than 5 g/cm^3 are often considered as heavy metals (Jarup, 2003). Metals are released into the marine ecosystem not only by natural sources such as volcanoes, natural weathering of rocks but also by numerous anthropogenic activities, such as mining, combustion of fossil fuels, discharge of industrial effluents, agricultural, domestic wastes etc. Basically, metals are separated into the essentials and non-essentials in classes A and B, and in a borderline class (Niebohr and Richardson, 1980).

- Class A: Calcium (Ca), Magnesium (Mg), Manganese (Mn), Potassium (K), Sodium (Na), Strontium (Sr)
- Class B: Cadmium (Cd), Copper (Cu), Mercury (Hg), Silver (Ag)
- Borderline: Zinc (Zn), Lead (Pb), Iron (Fe), Chromium (Cr), Cobalt (Co) Nickel (Ni), Arsenic (As), Vanadium (V), Tin (Sn).

Some metals, such as Fe, Mn, Zn and Cu are essential micronutrients but if their concentrations exceed the limit, they become harmful for the biota. Whereas, few heavy metals, such as Cd, Pb and Hg are toxic to the organism even in smaller quantities.

The seriousness of heavy metals contamination is compounded by the fact that they are generally persistent, water soluble, non-degradable, vigorous oxidizing agent. They tend to accumulate in high concentrations in organisms and occupying the higher trophic levels in food chain. The extent of bioaccumulation of metals is dependent on the total amount and the bioavailability of each metal in the environmental medium and the route of uptake, storage and excretion mechanisms in organism.

It is well proven that contaminant concentrations in water are typically low, often below detection limits, and can vary greatly over time and space, whereas, contaminants accumulate in sediments, and therefore provide a degree of time integration which is not found in water analysis (Rainbow and Phillips, 1993). However, it is important to note that both in sediments and in water contaminant concentrations determined by chemical analysis cannot be reliably used to assess the likely toxicity of contaminants to biota (Rainbow, 1995); aquatic organisms have been increasingly used in the assessment of contamination (Rainbow, 2002). Therefore, there is a need to demonstrate a link between exposure to and/or accumulation of a contaminant and their adverse, sublethal, biological response in organism.

1.9 Oyster as a Metal Bioindicator

Biomonitoring method based on bioindicators is one of the most appealing tool for the assessment of metal pollution in aquatic ecosystem. Aquatic invertebrates mainly bivalves are known to accumulate heavy metals in their tissue to a level above the ambient environment and yet survive in polluted environments (Dallinger and Rainbow, 1992; Rainbow, 2002). The rationale behind the use of bivalves in monitoring programs has been discussed in several scientific reports since the introduction of the 'Mussel Watch' programme in 1976 (Goldberg et al., 1978; Phillips, 1980; Farrington et al., 1983; O'Connor, 2001). Compared to other bivalves, oysters have exceptional bioaccumulation capacity which make them very suitable species for biomonitoring in marine waters (Lytle and Lytle, 1982; Mo and Neilson, 1993), and are widely used to determine the levels of metal in estuaries and coastal waters worldwide (Lytle and Lytle, 1982, 1990; Sadig and Alam, 1989; Peerzada and Kozlik, 1992).

1.10 Ecotoxicological Perspective

‘Ecotoxicology’ is defined as the ecological and toxicological effects of chemical pollutants on species populations, communities and ecosystems with the fate (transport, transformation and breakdown) of such pollutants in the environment (Forbes and Forbes, 1994). It shows a complex interrelationship between levels and type of chemical pollutant, living organisms within the environment and the environment itself (Sheehan et al., 1995). Coastal and estuarine ecosystems are one of the most polluted water bodies around the world due to high level of anthropogenic activities. Many of the pollutants adhere to tiny particles and find a way in planktonic and benthic animals, mostly in filter

feeders such as oysters. Heavy metals are one of the major pollutants and are responsible for long-term toxicological effects on marine ecosystems (Jarup, 2003).

The requirement of essential metals in different organisms vary substantially but optimal concentration ranges are narrow and frequently under homeostatic control. Excess metal concentration in an organism must be actively excreted, compartmentalized in cells or tissues, or metabolically immobilized (Coombs and George, 1978). Some metal escape all these actions causing toxic and other adverse effects (Chapman et al., 1996; Rand and Petrocelli, 1985). The impacts of such heavy metals have no visible influence compared to other pollutants. In fact, the toxic metals are concentrated and biomagnified upward within ocean food web at various trophic levels and eventually affecting human health (Bragigand et al., 2004).

1.11 Bioavailability of Metals

Since oysters are filter feeders, high levels of metal content enter into their body from their ambient environment. Organism bioaccumulate the metals in their tissues in proportion to the degree of environmental contamination from water, suspended particle matters, sediments and through food chains (Blackmore and Morton, 2001). Therefore, accumulated metal concentrations in an organism are a direct reflection of the bioavailability and contamination of that region. Nitrogen and sulfur seeking properties of metals contributes to their ability to form complexes with such centers in biological ligands in the tissue (Marigomez et al., 2002). Moreover, physico-chemical factors of the aquatic system such as salinity, dissolved organics, pH, hardness, sedimentary loads,

sediment texture, etc. influence the chemical forms of metals which are also responsible for the metal bioavailability and its toxicity to the organism (Rainbow, 1995).

1.11.1 Mode of Metal Uptake

The degree of metal accumulation is directly proportional to assimilation efficiency (AE) for metals which reveals the fraction of ingested elements that is merged into biological tissue (Wang and Fisher, 1999). Metal AEs in the oysters are higher than those measured in other bivalves such as mussels and clams (Ke and Wang, 2001). In oysters, quantity and quality of food supply has the highest effect on metal assimilation (Wang and Wong, 2003). Metals from the aquatic environment are bioaccumulated by oysters either passively from water or by facilitated uptake (Guo et al., 2001). The absorption of metals by oysters involves transfer of metals to the circulatory system across the epithelial barrier of gills and digestive gland (Roesijadi and Unger, 1993; Trombini et al., 2010). Endocytosis is used in assimilating metals from food in the digestive tract of invertebrates. In this process, particulate metals are most commonly ingested and then taken up after solubilization in the gut. Once endocytosed, the particulate metal complexes can be broken down and the metal redistributed to other intracellular ligands.

1.11.2 Cellular Storage and Sequestration of Metal

Sequestration of metals in an immobilized form occurs in various tissues and organs that are involved in pathways for metal uptake, transport, utilization and release. Sequestration in any tissue may be temporary or long term. The two most studied intracellular structures involved in metal sequestration, storage and excretion in oyster are

the metallothionein (MT) protein and intracellular vesicle-bound granules (Walker et al., 2006; Wang et al., 2011).

1.11.2.1 Metallothionein and Intracellular Granules

Metallothioneins (MT) are a family of low-molecular-mass peptides found mainly in the cytosol, lysosomes and in the nucleus. It is high in the amino acid cysteine which contains a thiol group (-SH). The thiol group enable MTs to bind heavy metals. MT_S are believed to function in the regulation of the essential metals Cu and Zn as well as the detoxification of these metals (Wallner-Kersanach et al., 2000) and non-essential metals such Cd and Hg (George et al., 1976; Kagi and Kojima, 1987). The capacity for metallothionein induction is highest in tissues that are active in metal uptake, storage and excretion (Wang et al., 2011). Moreover, marine bivalves contain a wide variety of membrane-bound intracellular granules, to which metals bind. Classified as either 'granules' or 'concretions', these structures are generally associated with the digestive or excretory tissues of invertebrates.

1.11.3 Internal Transport of Metals via Haemocytes

The transport and distribution of metals to the internal organs occur via circulatory systems of oysters (Sokolova et al., 2004, 2005). In the open circulatory system of oyster, the terms 'hemolymph' and 'haemocytes' are used to refer 'blood' and 'blood cells' respectively. Haemocytes (considered as primary vehicles) are highly mobile, phagocytic and capable of transferring their metal loads to other tissues through exocytosis (Cheng, 1990; Sarasquete et al., 1997; Gagnaire et al., 2004).

1.11.4 Excretion of Metals

Aquatic organisms utilize a variety of mechanisms to eliminate metals from their bodies. Overall it is a species-specific, organ/tissue-specific as well as metal and ligand-specific process. Additionally, physical and chemical parameters, such as temperature and salinity, may affect the rate of metal release in aquatic animals (Denton and Burdon–Jones, 1981; Dahlgaard, 1986). Turnover rate of release of metals that are adsorbed to external surfaces, complexed to external mucus or complexed to low-affinity intracellular and extra-cellular ligands sites can take hours, days and weeks. While the tightly bound metals include those that are sequestered by calcined concretions, MT, ferritin and ceruloplasm takes months, seasons or years to release. In an exception, oyster utilize diapedesis mechanism for metal excretion. In this processes metal bound haemocytes migrate from internal tissues through epithelial layers and then into either the gut lumen or the surrounding waters (Roesijadi and Robinson, 1994).

1.12 Toxicology of Heavy Metals

The oysters are sessile organism, and have limited capacity to avoid physical changes in its environment. They experience gradual and abrupt variations in environmental conditions that occurs over period of time. Long-term changes in surrounding environment have significant spatio-temporal effects on oysters. Moreover, metals are recognized as long-lasting pollutants in estuarine systems, frequently potentiating the occurrence of biological adjustments in the exposed organisms. Hence, aquatic organisms inhabiting metal-contaminated estuaries may present several physiological responses that

can be associated with the toxicokinetics and/or toxicodynamics processes. Toxicokinetics, the evaluation of metal accumulated in organisms, represent the temporal integration of uptake, transport, transformation, accumulation, half-life periods and excretion. On the other hand, toxicodynamics is related to the toxic effects of metals at the organ, cellular, and molecular levels (Nordberg et al., 2014). Many environmental pollutants (including metals) and/or their metabolites, exert their toxicity in marine organisms by increasing the generation of reactive oxygen species (ROS) within the cells (Winston and Di Giulio, 1991; Manduzio et al., 2005; Lesser, 2006).

1.13 Reactive Oxygen Species and Oxidative Stress

In normal metabolism of aerobic multicellular organisms, oxygen is used efficiently and constantly in various biochemical reactions. About 85-90 % of oxygen taken up by the cells are utilized by mitochondria where it is reduced in the electron transport chain (ETC) to produce ATP. However, incomplete reduction of about 1-3 % of total oxygen results in the formation of various oxyradicals (i.e. ROS). Therefore, mitochondrial ETC is considered as a principal site for generation of ROS (Loschen et al., 1974; Halliwell and Gutteridge, 2001). Other than mitochondria, ROS also formed in the cell organelles such as endoplasmic reticulum, micro bodies and cytosol. The molecular oxygen is stable with its two lone electrons of parallel spins. However, in metabolic processes breakage of covalent bond, addition of electron to molecules or removal of hydrogen by other radicals is responsible for ROS formation (Grotto et al., 2009). Although ROS has a strong reactivity, they are non-specific regarding their target molecules. They act as an electrophilic species or oxidant agents and attack on all cellular component present in

their vicinity such as protein, lipid and nucleic acids (Manduzio et al., 2005). Based on the presence of paired and unpaired electrons in the outer orbital shell, ROS are categorized as radicals and non-radicals. The unpaired electrons in ROS are the cause of its extreme reactivity. When two radical react, a stable covalent bond is formed. On other hand, when a radical reacts with non-radical, a new radical is formed. ROS is essential at low concentration for multiple physiological processes such as immunity (Golub and Desamps-Latscha, 1985), apoptosis (Sandstorm and Buttke, 1993; Shimizu et al., 1995) and cell differentiation (Tatla et al., 1999). The former category of oxyradicals includes superoxide radical ($O_2^{\bullet -}$), hydroxyl radical ($^{\bullet}OH$), hydroperoxyl radical ($ROO^{\bullet}$), alcoxyl radical ($RO^{\bullet}$).

The excess production of ROS results in oxidative stress in organism. This often occurs due to consequence of an imbalance between pro-oxidants and antioxidants levels in the cells and tissues (Regoli et al., 2002; Viarengo et al., 2007). This leads to enzyme inactivation, oxidation of proteins, peroxidation of lipids, damage to DNA and ultimately cell death (Winston and Di Giulio, 1991; Emmanouil et al., 2007). Recent studies have shown that metals, such as Fe, Cu, Cd, Cr, Pb, Hg, Ni and V exhibit to produce reactive oxygen species and results in oxidative cell damage (Stohs and Bagchi, 1995). Molecular mechanisms of heavy metal cytotoxicity include damage to plasma membranes, following binding to proteins and phospholipids, inhibition of Na, K dependent ATPases, inhibition of trans-membrane amino acid transport, enzyme inhibition, lipid peroxidation and oxidative DNA damage, depletion of antioxidant enzymes (such as glutathione) through the generation of ROS (Stohs and Bagchi, 1995; Leonard et al., 2004).

1.13.1 Lipid Peroxidation

Under oxidative stress, lipid peroxidation (LPO) occurs which is a free-radical-mediated chain of reactions that once initiated results in deterioration of polyunsaturated lipids (Cakmak and Horst, 1991; Korte et al., 2000). The •OH radical is a powerful initiator of lipid peroxidation. Polyunsaturated fatty acids are the target reactive molecule which have carbon-carbon double bond separated by methylene groups. This double bond weakens the carbon-hydrogen bond, and therefore, hydrogen gets more easily abstracted by a free radical. Once the abstraction of hydrogen atom by free radicals occurs, the radical is stabilized and known as lipid free radical (conjugated dienes). Conjugated dienes react with O_2 and forms a lipid peroxyl radical (ROO•), which abstracts electron from other lipid molecules (polyunsaturated fatty acid) resulting in lipid hydroperoxides and another lipid free radical (Grotto, 2009). Lipid hydroperoxides are stable until they encounter transition metals, such as Fe or Cu. These metals catalyze the generation of alkoxyl (RO•) and peroxyl (ROO•) radicals from lipid hydroperoxides, which then continue the chain reaction within the membrane and proliferate the damage throughout the cells. Chain breaking antioxidants inhibit this process by scavenging alkoxyl (RO•) and peroxyl (ROO•) radicals.

1.14 Antioxidant Defense System

To maintain ROS formation at low concentration, organisms have evolved adequate antioxidant defense mechanisms, which consist of both enzymatic and non-enzymatic components. Enzymatic antioxidants consist of superoxide dismutase (SOD), catalase

(CAT), glutathione-s-transferase (GST), etc. and non-enzymatic components include reduced glutathione (GSH), ascorbic acid (ASA) and β-carotene (Halliwell and Gutteridge, 2001). These biomarkers are the early indicators of changes in the morpho-functional state of bivalves under the effect of pollutants because a toxic effect will be apparent at the subcellular level, before it is noticeable at higher population levels.

1.14.1 Superoxide Dismutase (EC 1.15.1.1)

Superoxide dismutase (SOD) was first isolated from bovine erythrocytes by Mann and Keilin (1938) and further physiological function of Cu-Zn-SOD was confirmed by McCord and Fridovich (1969). Shortly thereafter, Fridovich and his coworkers identified two other type of SOD from *E. coli* one containing Mn (Keele et al., 1970) and the other containing Fe (Yost and Fridovich, 1973). SOD is a metalloenzyme that destruct the superoxide anion radicals ($O_2^{\bullet-}$) to hydrogen peroxide (H_2O_2) by dismutation. The enzyme catalyzes the following reaction:

$$\text{SOD-M}^{(n+1)+} + O_2^{\bullet-} \rightarrow \text{SOD} - \text{M}^{(N)+} + O_2$$

$$\text{SOD-M}^{(N)+} + O_2^{\bullet-} + 2H^+ \rightarrow \text{SOD-M}^{(n+1)+} + H_2O_2$$

$$\text{Net reaction: } O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$$

Where M= Cu (n=1), Mn (n=2) and Fe (n=2)

The enzyme has three major isoforms depending upon type of metal ions it contains as its cofactor. Cu-Zn-SOD is exclusively found in cytoplasm of eukaryotic cells. Bovine Cu-Zn-SOD is dimeric and has molecular mass of 35 kDa. In the active site of Cu-Zn-SOD, Cu ion is coordinated by four histidine residues (His $_{44}$, His $_{46}$, His $_{61}$, and His $_{118}$) and a

water molecule (Tainer et al., 1982). The Zn ion is held by three histidine (His $_{61}$, His $_{69}$, His $_{78}$) and one aspartate (ASP $_{81}$) residues. The optimum pH is 5.3 to 9.5 and the rate constant is about 1.6×10^9/M/s. Mn-SOD is predominantly found in mitochondria (Fridovich, 1975). Eukaryotic Mn-SOD is tetrameric and has a molecular mass of 88 kDa. Unlike Cu-Zn-SOD, the activity of Mn-SOD is pH dependent. At pH 7.8 the rate constant is 1.8×10^9 /M/s and at pH 10.2 rate constant is about 0.33×10^9/M/s. Fe-SOD is found in bacterial cells. It is dimeric and has molecular mass of 44 kDa (Beyer et al., 1991). Like Mn-SOD, Fe-SOD activity is inhibited at high pH.

Apart from these three SODs, an extracellular SOD (EC-SOD) has been reported from extracellular space of vertebrates and invertebrates (Hjalmarsson et al., 1987; Tang et al., 1994; James et al., 1994). EC-SOD is dimeric and has molecular mass of 68 kDa (Carlsson et al., 1996). Recently, EC-SOD was detected in oyster plasma by Gonzalez et al. (2005). The activity of SOD can be inhibited by various substances e.g. Cu-Zn-SOD is inhibited by cyanide (Weisiger and Fridovich, 1973) whereas sodium dodecyl sulphate inhibits the activity of Mn-SOD. H_2O_2 has been found to hamper the activity of both Cu-Zn-SOD and Fe-SOD (Yim et al., 1990). In addition to the cytoplasm, SOD is found in the peroxysomes, lysosomes, external faces of the endothelial cells and core of the eukaryotic cells (Beyer et al., 1991). Deficiency in SODs and their inhibition increases the sensitivity of the organism to the oxidants (Manduzio et al., 2005).

1.14.2 Catalase (EC 1.11.1.6)

Dismutation of $O_2^{\bullet-}$ by SOD generates H_2O_2, which is toxic to cells. Cells are equipped with two principal H_2O_2 scavenging enzymes viz., catalase (CAT) and glutathione peroxidase (GPx). Catalase breaks down H_2O_2 to water and molecular oxygen. Reaction mechanism of CAT is as follows:

$$\text{CAT-Fe (III)} + H_2O_2 \rightarrow \text{CAT-Fe (IV) O} + H_2O$$

$$\text{CAT-Fe (IV) O} + H_2O_2 \rightarrow \text{CAT-Fe (III)} + H_2O + O_2$$

$$\text{Net reaction: } 2H_2O_2 \rightarrow 2H_2O + O_2$$

Catalase is a ubiquitous enzyme having a haeme group (Fe III) in its active site (Chance et al., 1979). It is tetramer and has molecular weight of 225-250 kDa. It is located primarily in peroxisomes and has very high Km (25 mM) value for its substrate (H_2O_2) which makes it most efficient at scavenging high concentration of H_2O_2 (Lehninger et al., 1993). In addition, a Manganese containing catalase (Mn-CAT) in *Lactobacilius plantarum* has been reported (Beyer and Fridovich, 1985). The molecular mass of the Mn-CAT is 172 kDa. It contains five subunits and each subunit contains Mn (III) ion. The reaction mechanism is as follows:

$$\text{CAT-Mn (III)} + H_2O_2 + 2H^+ \rightarrow \text{CAT-Mn (V)} + 2H_2O$$

$$\text{CAT-Mn (V)} + H_2O_2 \rightarrow \text{CAT-Mn (III)} + 2H^+ + O_2$$

$$\text{Net reaction: } 2H_2O_2 + 2H^+ \rightarrow 2H_2O + O_2$$

The activity of catalase is inhibited by azide, cyanide and aminotriazol. Among them, aminotriazol is the potent inhibitor of catalase activity (Darr and Fridovich, 1986). Unlike

haeme catalase, manganese catalase is not inhibited by azide or cyanide (Halliwell and Gutteridge, 2001).

1.14.3 Gluathione-S-Transferase (EC 2.5.1.18)

Gluathione-S-transferase (GST) catalyzes the conjugation and detoxification of many xenobiotics containing electrophilic centers (Mannervik and Danielson, 1988; Mannervik, 1985). The reaction mechanism of the enzyme is as follows:

$$RX + GSH \rightarrow RSG + HX$$

GSH conjugates are usually more soluble than the original compounds, and are removed from cells by membrane-associated ATP binding cassette transporter (Garcera et al., 2006). The GST_S (mammalians and non-mammalian origins) are multigene family of enzymes, which have been grouped into number of classes: Alpha, Beta, Delta, Epsilon, Kappa, Lambda, Mu, Phi, Pi, Sigma, Tau, Theta, Omega, and Zeta.

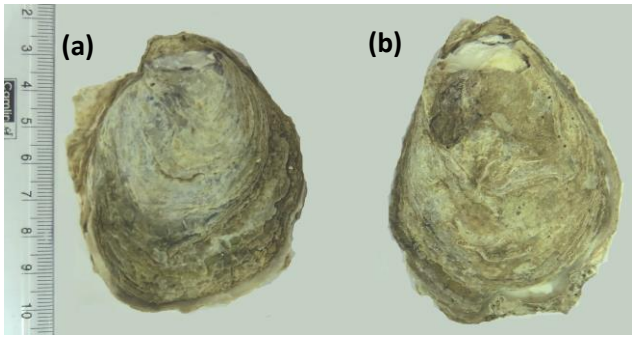

Plate 1.1 External view of (a) *Crassostrea madrasensis* (b) *Crassostrea gryphoides*

Plate 1.2 Internal view *Crassostrea madrasensis* (Preston, 1916).

Plate 1.3 Internal view *Crassostrea gryphoides* (Schlotheim, 1813).

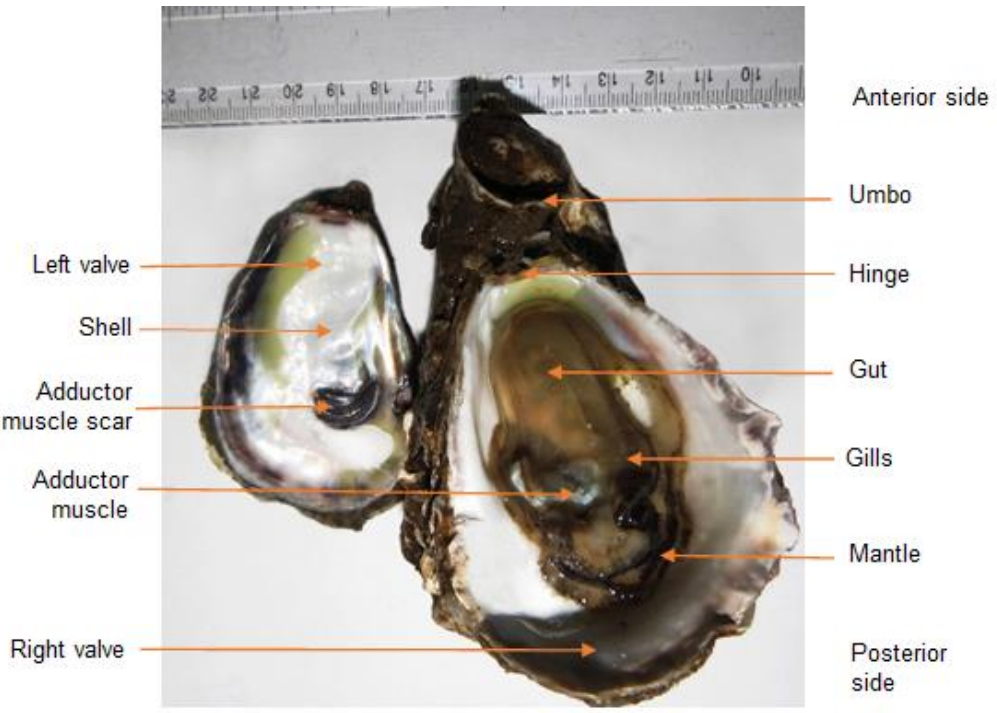

Plate 1.4 Anatomy of oyster.

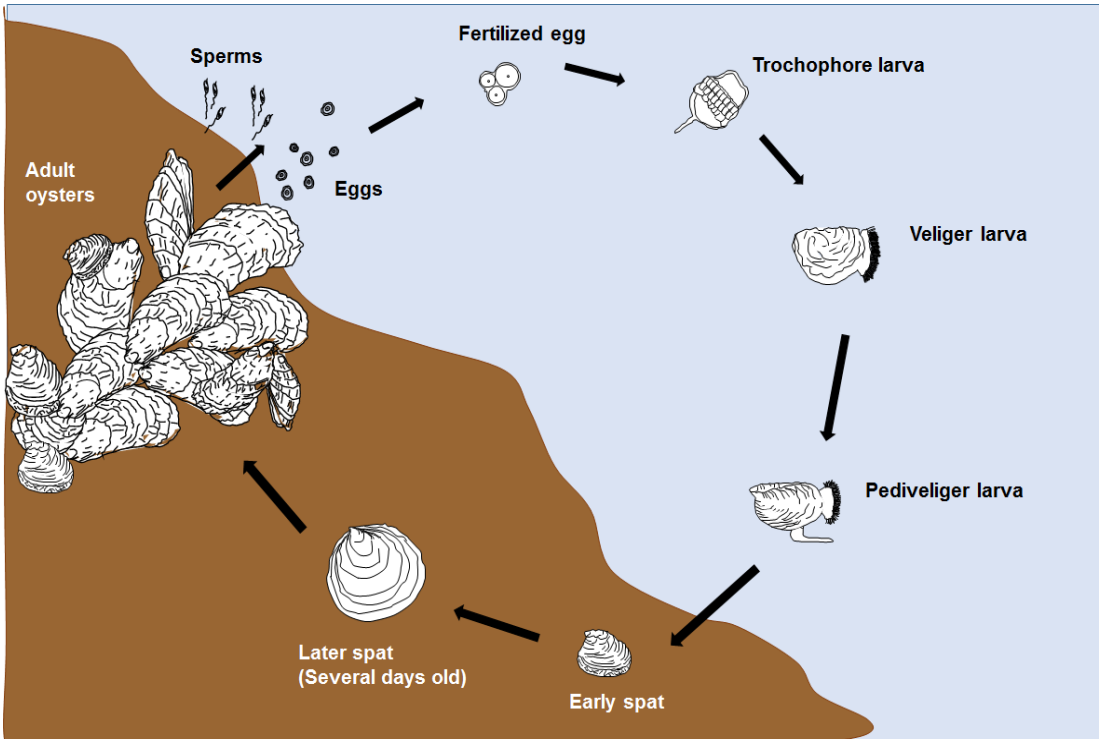

Figure 1.1 Life cycle of oyster.

Chapter 2

Review of Literature

Chapter 2

2.1 Concept of Ecotoxicological Studies

Study on ecotoxicology integrates ecology, environmental chemistry, biochemistry, and toxicology in a multidisciplinary manner. Increasing metal contamination of the coastal environment from both point and non-point sources, especially in developing countries attract the attention of environmental researchers to study ecotoxicology aspect locally and internationally (Shulkin et al., 2003). Basically, ecotoxicological tests include evaluations under both field and laboratory conditions (Calow, 1993). Field evaluations generally include species diversity indices, body size and population abundances of the organism whereas, laboratory experiments consider several possible methods by which interaction of toxic substances with organisms is evaluated. Standard laboratory bioassays include both acute and chronic tests (Moriarty, 1983). In general, two different approaches are used for toxicity testing such as embryological and somatic approach. Embryological approaches require expertise to identify different developmental stages, which required more frequent monitoring (sometime every 3-6 hrs durations) (Calabrese et al., 1973; Thain, 1991; Geffard et al., 2003). Whereas, somatic growth determinations may be faster but may cause death of experimental individuals due to inappropriate handling (Mallia et al., 2006). Indeed, since most of our concerns relate to human or ecosystem health, chemical and physical measurements of the environment is must therefore, be able to predict biological effects at several different levels of mature organism from field. Ecotoxicologists now a day are more concerned with identifying the effect of various contaminants on the organism by analyzing different biological variables which are sensitive to toxicant stresses.

One way to achieve this is through biomarkers that induce the antioxidant defenses in cells of the organisms (Livingstone, 1993). Biomarkers are being increasingly recognized as cost-effective tool in ecotoxicological studies to provide more accurate information on *in situ* toxic effects of pollutants on marine organisms before they reach the population or community levels (Winston and Di Giulio, 1991; Lagadic et al., 1994; Regoli et al., 1998; Brown et al., 2004; Cotou et al., 2013). There are various biomarkers such as superoxide dismutase (SOD), catalase (CAT), glutathione-s-transferase (GST), glutathione reductase (GR), glutathione peroxidase (GPX) which are being used in the assessment of biological effect of contaminants including metals (Ringwood et al., 1999a; Andersen et al., 2006; Cong et al., 2013; Vazquez-Boucard et al., 2014).

2.2 Metals Accumulation in Oysters from International Waters

Oysters have already been proposed as sentinel organisms for marine ecotoxicological studies for assessing marine environmental health status, because they tolerate heavy metals to high level in their body and provide a time-integrated estimate of bioavailable metal concentrations (His et al., 1999; Cruz-Rodriguez and Chu, 2002). Several studies around the globe have been undertaken in relation to metal contamination in oysters in past few decades (Phillips, 1979; Denton et al., 1999; Silva et al., 2001; De Mora et al., 2004; Azlisham et al., 2009; Chen et al., 2014). Results of several investigations have suggested that the oyster is useful as a biomonitoring organism to continually trace current metal levels in the estuarine and coastal regions of tropical and subtropical waters (Table 2.1). However, metal accumulation in oysters highly depends on various endogenous and exogenous factors (Frazier, 1975). For example, Milazzo et al. (2014)

showed that metal accumulation in *Crassostrea rhizophorae* varied depending on the seasonal variation in biochemical composition and the bioavailability of metals in both water as well as in sediment.

2.2.1 Influence of Ambient Metal Concentrations on its Accumulation in Oysters

It has been recognized that the increasing release of anthropogenic waste into the marine environment affects the composition of water and sediments (Apeti et al., 2005b; Balkis et al., 2010; Chen et al., 2014). Dassenakis et al. (1995) suggested that approximately 90 % of the suspended matter of rivers, which are main carriers of metals, settles in estuaries and the coastal zone. The concentrations of metals from ambience further affect the flora and fauna that could prove detrimental to marine life (Watling and Watling, 1976). Thus, the analysis of water and sediments, to assess the degree of metal contamination in marine environments, has become widespread practice throughout the world (Dassenakis et al., 1995; Shulkin et al., 2003; De Mora et al., 2004). This area of study is highly relevant and considered to the ecotoxicology field as it led to the adoption of the bioindicator concept for the environmental quality assessment (Catsiki and Florou, 2006; Alfonso et al., 2013).

2.2.1.1 Influence of Metal Concentrations in Sediment

Quantitatively, sediments represent the major compartment for metal storage in aquatic environments (Gagnon and Fisher, 1997). However, increase in concentration in sediments not necessarily mean that there will be higher accumulation in tissues. Lee et al. (1998) noticed that the seasonal variation of the Cu concentration in surface sediment

from Ann-ping (Taiwan) did not show an increasing trend as observed in oyster tissue from 1993-1996. Relationships of Cu, Pb, Zn, Cd, Cr, Fe and Mn concentrations between sediment and tissue of *C. virginica* and shells have been studied from Gulf of Mexico estuaries (Huanxin et al., 2000). Authors concluded heavy metal accumulation in oyster was dependent on biogeochemical properties and the oyster physiology as well as metal concentration in water and sediment. Geffard et al. (2003) studied bioavailability and toxicity of sediment Cu, Cd and Zn in *C. gigas* embryos and larvae and found that sediment associated metals cause the abnormality in larval development of oyster. Hedouin et al. (2009) found that sediment was the dominant pathway for Cd accumulation in oysters. Likewise, Birch and Hogg (2011) reported strong positive correlation between the Cu concentration in fine sediment and in oyster tissue. Shirneshan and Bakhtiari (2012) also observed positive correlation between Cu and Cd content in sediment and in *Saccostrea cucullata* tissue as well as Pb levels in sediment and oyster shell from Qeshm Island in the Persian Gulf. Fuad et al. (2013) found significant correlation between relative enrichment of Cu, Pb and Zn in *S. cucullata* and rare earth elements which explains that metals derived from natural sources. However, Birch et al. (2014) found poor correlation among metal concentrations in surface sediment, suspended particulate matter and in oyster tissue.

2.2.1.2 Influence of Dissolved and Particulate Metal Concentrations

Various researchers have examined the correlation between metal concentration in the oyster and in the ambient waters (Zamuda and Sunda, 1982; Marcintic et al., 1986; Hung and Han, 1990). Oysters as a filter feeders take up metals directly from sea water (in form

of dissolved ions and colloidal particles) (Guo et al., 2001, 2002). Guo et al. (2001) examined the effect of dissolve organic carbon (DOC) on metal bioavailability and its uptake by *C. virginica* using radiotracer technique. They observed increase and decrease in metal uptake was directly proportional to the DOC concentration. Based on the results authors (Guo et al., 2001) suggested that the bioavailability and toxicity depends on the types of metals and interaction of metal with biological organic ligands. In another study, Brooks et al. (2007) found labile Cu (free ions and inorganic complexes) was responsible for toxicity to the oyster embryo. Hedouin et al. (2010) suggested that metals present in dissolved phase gets more readily bioconcentrated (3-4 times) than the metal present in the sediment. Alfonso et al. (2013) reported high concentrations of metals (Cd, Cu, Ni and Mn) in *C. rhizophorae* from the Buche estuary occurred due to atypically raised bioavailable forms of these metals.

Suspended particulate (phytoplankton and inorganic resuspended sediment particles), is the important food source of the oyster and, play an important role in transportation and bioaccumulation of metal in oysters (Zamuda and Sunda, 1982; Rainbow, 1995; Lee et al., 1996; Hedge et al., 2009). For example, Apeti et al. (2005b) found that metal concentrations in particulate phase correlate significantly with concentration in the tissue of *C. virginica* than those in the sediment. Whereas, Robinson et al. (2005) found Cu concentrations in *S. glomerata* from several estuaries in New South Wales occurred via both dissolved and particulate pathways. Oysters are known to accumulate very high concentrations of Cd, Cu, and Zn in their tissues due to their exceedingly high dissolved uptake and dietary assimilation efficiency. Ke and Wang (2001) studied the metal uptake

in estuarine and coastal oysters and found that the estuarine oyster *C. rivularis* (*C. hongkongensis*) can absorb up to 75 % of Cd and 80 % of Zn from phytoplankton.

2.2.2 Influence of Other External (Ecological) Factors on Metal Accumulation in Oysters

The accumulation of metals vary through time, mainly due to changes in physico-chemical variables, such as water temperature and salinity (Abbe et al., 2000; Bryan and Langston, 1992). For example, Belivermis et al. (2016) investigated the combined exposure effects of three different pH (7.5, 7.8 and 8.1) and two different temperatures (21°C and 24 °C) on the *C. gigas* under dissolved radiotracers ^{109}Cd in laboratory conditions. They found that at pH 7.5, Cd was accumulated with an uptake rate constant two-fold higher at 24 °C than 21 °C in oyster. Previously, Aguilar et al. (2012) found that the physiological stage of the oyster, the high content of finer particles (silt and clay) and amount of organic matter in sediment have a significant positive influence on the transport and availability of heavy metals in oyster.

2.2.3 Influence of Internal (Biological) Factors on Metal Accumulation in Oysters

Bioaccumulation of various metals by oysters vary seasonally in the tissues as well as in the shells. It depends on biological factors such as age (size) of oysters, sex, reproductive cycle stage, gonadal development and spawning phase (Fraziar, 1975; Boyden and Phillips, 1981; Paez-Osuna and Marmolejo-Rivas, 1990; Paez-Osuna et al., 1995; Frias-Espericueta et al., 1999). For example, during spawning period, loss of body weight and decrease in metal levels in tissue have been recorded in *C. virginica* (Zaroogian, 1979;

Zaroogian, 1980). Moreover, distribution of metals in different organs (Okazaki and Panietz, 1981; Jing et al., 2006); ploidy level (diploid and triploid) (Amiard et al., 2005) influences the concentration of the metals in the oysters. Bray et al. (2015) and Paez-Osuna and Osuna-Martinez (2015) observed the species-specific difference in metal concentrations due to the difference in reproductive stages of both the oyster species.

2.2.4 Metal Toxicity in Oyster

Although the total metal concentrations in sediment, water and organisms give a convenient measure of metal pollution and bioavailability, such measures do not necessarily predict the toxicity of these pollutants to aquatic organisms (Luoma, 1996; Amiard et al., 2008). Effects on organisms can be traced to molecular and cellular level responses, as pollution impact does not necessarily lead to observable effects on a population or ecosystem level (Catsiki and Florou, 2006).

The earliest reports on effects of heavy metals can be traced back to 1880 in France (Lankester, 1886). He observed the green colour oyster due to Cu accumulation in their tissue taken up from surrounding old copper mines and copper-bottomed ships. Green oysters also have been documented in several estuaries and coasts around the world, including southwest England (George et al., 1978) and Spain (Sarasquete et al., 1997). Sarasquete et al. (1997) noticed inflammatory lesions to granular amoebocytes in green colored *C. angulata* due to presence of high concentration of Cu and Zn. In Asia, first case of green oysters due to higher Cu accumulation was observed in Taiwan in January 1986 with a mass mortality (Hung and Han, 1990; Han and Hung, 1990; Lee et al., 1998).

Metal pollutants also have deleterious effects upon the fertility of oyster. Several researchers (Conner, 1972; Calabrese et al., 1973; Calabrese et al., 1977; Martin et al., 1981) have worked on various metal toxicity (LC_{50} test) aspect on embryonic and larval stages of oyster. They found that the metals were more toxic at embryonic and larval stages than adults of the same oyster species. Watling (1978) reported extreme toxicity of Cd to *C. gigas* larvae and spat by reducing their number of settling and viability. Exposure to heavy metals in adult oysters cause a stress on gametes and disturbance in embryonic development (Zaroogian and Morrasson, 1981). Ringwood and Conners (2000) observed that parental depletion of GSH increases the susceptibility in developing embryo to metal (Cd and Cu) toxicity. Under Cu and Cd exposure decrease in haemocyte and granulocyte count and decrease in the total immunity of the oyster were observed by Cheng (1988, 1990) and George et al. (1983). It has been proved that exposure to metals accumulation results in destabilization and damage of the lysosomal membranes in oysters (Ringwood et al., 1999a; Ringwood et al., 2002). Furthermore, Shuster and Pringle (1969) and Engel (1999) demonstrated that whole body concentration of Cd more than 100 µg/g wet wt. resulted in death of the organism. Therefore, this indicates that the detoxification system of oyster has limit for Cd accumulation. Sokolova et al. (2005) studied Cd distribution in different subcellular fractions of gill and hepatopancreas tissues of *C. virginica* under the exposure of 25 µg Cd/l (low sublethal concentration) for up to 21 days. These authors noticed dysfunction of mitochondria (reduce the ATP generation) and lysosomes. In another study, exposure to Cd caused weight loss, deterioration of tissue, and finally death of oyster (Barrera-Escorcia and Wong-Chang, 2005). Similarly, Pb accumulation in pearl oyster *Pinctada imbricata* has shown reduction in growth of the

organism (MacFarlane et al., 2006). Histological analysis of oysters having Pb and Cd concentration above the permissible limit in *C. virginica* showed histopathological lesions in 47 % of the oysters studied (Guzman-Gracia et al., 2009). Barrera-Escorcia et al. (2010) found depletion in filtration rate, food assimilation and assimilation efficiency capacity with weight loss and mortality in *C. virginica* from Mandinga Lagoon, Mexico under external Cd concentration (170 μg Cd/l). Aguilar et al. (2012) have observed that though the *C. virginica* has high tolerance capacity for heavy metals, but the Cu decreased the condition index of oyster. Ji et al. (2014) showed that high concentration of Cu, Ni, Pb and Cd in gills disturbed the osmotic regulation and metabolic pathways of *C. hongkongensis*. Luo et al. (2014) suggested that exposure to heavy metals contamination significantly suppress the stress and immunity response system of oysters. High percentage of abnormal growth were observed in *C. gigas* larvae (D-shape larvae) from Archachon Bay (France) when exposed to 1 μg Cu/l.

2.2.5 Antioxidant Defense Mechanism against Metal in Oyster

Various antioxidant biomarkers (SOD, CAT, GST, GR, GPX, LPO, etc.) have been studied in whole tissue and/or body parts of oysters against various conditions such as larval growth of oyster (Genard et al., 2011); variation in environmental gradients (e.g. salinity and temperature) (Damiens et al., 2004); diesel oil (da Silva et al., 2005); sewage discharge (Zanette et al., 2008), metals (Andersen et al., 2006; Ivanina et al., 2008; Choi et al., 2008; Cong et al., 2013). In oysters, metals are reported to induce oxidative stress where antioxidant defense system can provide the special protection against the stressors.

2.2.5.1 Field Observations

Ringwood et al. (1999b) noticed depletion in GSH and increase in LPO activities in juvenile *C. virginica* collected from various metal contaminated sites compare to reference site. Funes et al. (2006) observed higher activation of CAT, SOD and GPX in *C. angulata* samples collected from the South Atlantic Spanish littoral which had high level of Cu and Zn accumulation in their tissue. Another study by Andersen et al. (2006) reported increased LPO activity with increase in metal concentrations in oyster. Lannig et al. (2006) reported synergetic effect of temperature and Cd with low condition index, elevated LPO and high mortality whereas with elevated temperature with no Cd showed no significant variation in condition index, survival rate and LPO in *C. virginica*. Similar results were obtained in *C. virginica* where mitochondrial respiration with more sensitivity under high atmospheric temperature and increasing Cd accumulation were reported (Cherkasov et al., 2010). Luna-Acosta et al. (2010) noticed significant differences in SOD (in gills and digestive gland) in juvenile *C. gigas* between metal contaminated and reference sites and concluded that enzyme activity depends on the body compartment, the season, and/or the site. Vazquez-Boucard et al. (2014) noticed positive correlation among GST activity, genotoxic damage and Ni content in the oysters.

2.2.5.2 Laboratory Experiments

The results of previous laboratory studies with oysters (*C. virginica*) suggested that embryos from GSH-depleted parents are more susceptible to metal toxicity (Ringwood and Conners, 2000). Glutathione was depleted when *C. virginica* exposed to Cu in laboratory condition and susceptible to adverse potential toxicity (Conners and

Ringwood, 2000). Geret et al. (2002) found a decrease in MDA levels with an increase in MT levels in gills of *C. gigas* which was exposed to Cd and Cu for 21 days. Andersen et al. (2006) observed initial induction of GSH and GST followed by declined in these enzyme levels after certain period of Cu exposure. Further restimulation of response for CAT and GST during the depuration phase indicating increase in production of two enzymes indicates detoxification or depuration of Cu from the cells. Jing et al. (2006, 2007) reported significant increase in SOD activity in the mantle of the pearl oyster *Pinctada fucata* after exposure to sublethal Pb and Cu for 24 hrs and trend was downwards up to 72 hrs. Jo et al. (2008) cloned CAT and GPX cDNA and investigated its time- and dose-dependent effects against Cd concentrations (0.01, 0.05 or 0.1 mg/kg) on mRNA levels of antioxidant enzymes SOD, CAT, GPX in *C. gigas*. They observed in 0.1 mg/kg Cd exposed oyster mRNA expression of antioxidant enzymes increased in initial phase and decrease in later part of experimental period. These results suggest that antioxidant enzymes play important roles in the physiological changes related to metabolism and cell protection that occur in *C. gigas* exposed to Cd. Wang et al. (2015) observed simultaneous variation in activity of CAT by modulating reactive oxygen species (ROS) metabolism with temperature and Cu level in *C. ariakensis*. Barrera-Escorcia and Wong-Chang (2010) observed increased LPO and MT levels in *C. virginica* tissue (digestive gland, adductor muscle and gills) after 6 hrs and 48 hrs of exposure to Cd.

2.3 Metals Accumulation in Oyster from Indian Coast

Studies on metal accumulation in oyster have been carried out at the Bombay coast (Bhatt et al., 1968; Krishnakumari et al., 1992); Goa coast (Zingde et al., 1976; Nagi, 2008; Chakraborty et al., 2016); Karnataka coast (Krishnakumar et al. 1998); Cochin coast (Sankaranarayanan et al., 1978); Deltaic Sundarbans (Sarkar et al., 1994); Pulicate lake (Laxmi Priya et al., 2010); Andhra Pradesh coast (Gawade et al., 2013); Andaman Island waters (Abhilash et al., 2013) (Table 2.2). *Crassostrea madrasensis* and *C. gryphoides* have been used with considerable success in metal contamination monitoring programmes in India (Mitra et al., 1987; Rajendran et al., 1988; KrishnaKumar, 1990; Bhattacharya et al., 1994; Sarkar et al., 2008). Sankarnarayana et al. (1978) reported high metal accumulation in *C. madrasensis* during non-monsoon period from Cochin coast due to less fresh water flow and influence of industrial and domestic waste. On the contrary, Senthilnathan and Balasubramanian (1998) observed high metal concentrations in same oyster species in monsoon than non-monsoon season. They suggested that higher metal accumulation was due to the more influx of metal rich freshwater as well as more availability of metal associated particulate matter along with suspended sediment in monsoon season. Biswas et al. (2013) and Yesudhason et al. (2013) found that metal accumulation in *S. cucullata* is positively correlated with temperature, salinity and pH. The results mentioned in these studies is due to the upwelling event which occurs in end of summer season driven by strong southwest monsoonal wind and bring nutrient and metal rich water to surface. Furthermore, Rajendran et al. (1988) and Mitra et al. (1993) found gill and mantle exhibited higher concentration of metals than the other soft tissues of *C. madrasensis*. Raghavan et al. (2003) also noticed that the concentrations of heavy

metals in tissue of *C. madrasensis* corresponds to the level of those metal concentration in seawater and sediment. However, Chakraborty et al. (2016) suggested that bioaccumulation of Cd in *Crassostrea* sp. along the Goa coast depends not on the total Cd concentration in sediment but on the speciation of Cd in the marine system.

2.3.1 Metal Toxicity in Oyster

Ittoop et al. (2006) observed extensive damage to the adductor muscles, mantle and gills with maximum impairment in epithelial cells of tissue in Cu treated (0.5 and 1 mg/kg at salinity 12) oysters. In addition, the impairment in filtration rate and pumping activity of the oyster in higher Cu concentration has been observed. In the same experimental setup, Ittoop et al. (2009) also observed the decrease in haemocyte and granulocyte count and impaired the immunity in oysters. Kumaraguru and Ramamoorthy (1978) recorded mortality of *C. madrasensis* under Cu toxicity experiment.

2.3.2 Studies on Antioxidant Defense Mechanism in Oyster

Jana et al. (2013) studied the seasonal variations in antioxidant enzyme activity and lipid peroxidation in *S. cucullata* and noticed high enzyme activities and lipid peroxidation in response to high metal concentrations in Sundarban waters.

So far, oysters in relation with metal pollution and ecotoxicological studies have been studied in various regions of the world. Historically, the Indian oysters are one of the most important and promising economic oyster species being harvested for human consumption, and is frequently exposed to multiple stressors in the environment.

Compared to the studies conducted in other region of the world, reports on the metal accumulation and antioxidant enzyme responses against it are totally lacking in oyster species which are predominantly inhabiting the Indian waters.

Table 2.1 Heavy metal (Cu, Ni, Pb and Cd) concentrations (average or ranges of average, mg/kg) in whole soft tissue of oysters reported from international waters (year 2001–2016).

Oyster species	Location	Dry/wet wt.	Cu	Ni	Pb	Cd	References
Crassostrea gigas	Huludao Coast	Dry	474	2.5	5.9	615	Gao et al., 2016
Crassostrea sp.	Setiu Wetlands, Malaysia	Dry	0.45 – 2.44		0.13 – 0.38	0.05 – 0.31	Shaari et al., 2016
Ostrea edulis	Adriatic sea, Croatia	Wet	10.3 – 55.6	Nr	0.22 – 0.54	0.66 – 1.20	Bilandzic et al., 2015
Crassostrea gigas	North sea, Kent	Dry	391	Nr	1.1	2.2	Bray et al., 2015
Crassostrea corteziensis	SE Gulf of California	Dry	60	Nr	1.11	6.05	Paez-Osuna and Osuna-Martinez, 2015
Crassostrea angulata	South west Taiwan	Dry	330.3	Nr	3.6	1.7	Chen et al., 2014
Saccostrea cucullata	Gulf of Chabahar, Oman sea	Dry	59.25 – 145.1	15.4 – 38.0	2.36 – 17.5	0.08 – 0.45	Bazzi, 2014
Crassostrea sp.	Musa estuary, northwest Persian Gulf	Dry	207.2 – 403.5	Nr	0.07 – 0.95	12.21 – 17.5	Sarmadian et al., 2014
Crassostrea rhizophorae	Western coast of Trinidad	Wet	4.2 – 12.3	0.1 – 5.5	0.1 – 0.9	0.1 – 0.2	Kanhai et al., 2014
Saccostrea cucullata	Hendourabi Island-Persian Gulf, Iran	Dry	Nr	Nr	0.67	63.3	Salahshur et al., 2014
Crassostrea gigas	Mexico coast	Dry	32.8 – 141.3	0.1 – 6.2	7.2 – 9.9	4.2 – 7.3	Vazquez-Boucard et al., 2014
Saccostrea cucullata	Oman Coast of the Arabian Sea	Dry	Nr	Nr	0.01	3.41	Al-Ghassani et al., 2013
Crassostrea rhizophorae	Venezuela coast	Dry	16.8 – 83.0	1.9 – 16.9	2.3 – 3.5	1.5 – 4.2	Alfonso et al., 2013
Saccostrea cucullata	East coast of Peninsular Malaysia	Dry	84.12 – 238.2	Nr	0.64 – 4.38	0.99 – 2.64	Fuad et al., 2013
Saccostrea glomerata	Sydney estuary, Australia	Dry	174 – 4606	Bd – 4.4	1.3 – 18	Bd – 5.9	Birch et al., 2014
Saccostrea cucullata	Qeshm Island, Persian Gulf Iran	Dry	32.9 – 282.6	Nr	0.08 – 0.60	1.01 – 2.33	Shirneshan et al., 2013
Saccostrea cucullata	Persian Gulf, Iran	Dry	294.1 – 345.8	Nr	20.6 – 58.2	10.3 – 12.0	Heidari et al., 2013
Crassostrea gigas	Ebro Delta in Catalonia, Spain	Dry	38.8 – 98.7	Nr	0.26 – 0.78	0.50 – 1.32	Ochoa et al., 2013
C. hongkongensis	Pearl River Estuary, China	Dry	511 – 4966	Nr	1.1 – 2.3	6.5 – 23.2	Yu et al., 2013

Table 2.1 …Continued…

Table 2.1 …Continued…

Oyster species	Location	Dry/wet wt.	Cu	Ni	Pb	Cd	References
Crassostrea virginica	Gulf of Mexico	Dry	47.1 – 231.1	Nr	Nr	0.4 – 4.30	Aguilar et al., 2012
C. virginica	San Andres Lagoon, Mexico	Dry			0.73 – 0.86	2.21 – 2.33	Vazquez-Sauceda et al., 2011
Crassostrea virginica	Southwest Louisiana	Dry	58 – 245	Nr	1.6 – 5.8	4.0 – 6.3	Siva et al., 2010
Crassostrea iredalei	Setiu Lagoon, Malaysia	Dry	40.1	Nr	1.5	2.42	Azlisham et al., 2009
Crassostrea virginica	Mandinga Lagoon, Veracruz, Mexico	Dry	Nr	Nr	<5.84	2.23	Guzman-García et al., 2009
Isognomon isognomon	SW lagoon of New Caledonia	Dry	3.1 – 17.3	2.2 – 16.0	Nr	1.18 – 1.80	Hedouin et al., 2009
Crassostrea sps.	Urdaibai Estuary, Spain	Dry	29.6 – 143.7	1.62 – 9.2	0.63 – 3.81	0.41 – 3.0	Raposo et al., 2009
Crassostrea virginica	USA Coast	Dry	Nr	Nr	Nr	1.1 – 9.9	Apeti et al., 2009
Crassostrea corteziensis	Coastal Lagoon of NW Mexico	Dry	71.5	Nr	8.36	6.47	Frias-Espericueta et al., 2008
Crassostrea iredalei	East coast Peninsular Malaysia	Dry	38.9	Nr	0.17	1.6	Najiah et al., 2008
Crassostrea virginica	Savannah estuary, USA	Dry	67 – 120	<1.5 – 2.5	<1.5 – 4.0	<1.5 – 2.9	Senthil et al., 2008
Saccostrea cucullata	Clear water bay, Hong Kong	Dry	480	Nr	Nr	3.1	Wang and Wong, 2006
Ostrea stentina	Iskenderun Bay, Turkey	Dry	64.7	6.92	6.21	4.27	Turkmen et al., 2005
Saccostrea cucullata	Gulf and Gulf of Oman	Dry	60.9 – 276	0.80 – 3.14	0.25 – 0.67	6.2 – 21.9	De Mora et al., 2004
Crassostrea rhizophorae	Rio de Janeiro, Brazil	Dry	Nr	Nr	Nr	1.3 – 5.4	Rebelo et al., 2003
Crassostrea iridescens	Mazatlan Bay, SE Gulf of California	Dry	86.9	5.41	2.3	2.3	Soto-Jimenez et al., 2001
Crassostrea rhizophorae	Potengi estuary, Brazil	Dry	14 – 353	0.4 – 7.4	0.6 – 26.1	0.4 – 4.2	Silva et al., 2001

Nr = Not reported, Bd = Below detectable limit

Table 2.2 Heavy metal (Cu, Ni, Pb and Cd) concentrations (average or ranges of average, mg/kg) in whole soft tissue of oysters reported from Indian coast.

Oyster species	Location	Dry/wet wt.	Cu	Ni	Pb	Cd	References
Saccostrea cucullata	Andaman Island	Wet	0.72 – 154.11	0.44 – 4.08	2.99 – 8.35	Nr	Abhilash et al., 2013
Crassostrea madrasensis	Andhra Pradesh coast	Dry	Nr	20	13.5	10.4	Gawade et al., 2013
Saccostrea cucullata	West Bengal coast	Dry	106.9 – 726.81	Nr	5.34 – 31.36	5.3 – 37.01	Biswas et al., 2013
Crassostrea madrasensis	Pulicate lake	Wet	47.84 – 67.18	5.17 – 6.18	12.76 – 13.48	0.78 – 0.91	Laxmi Priya et al., 2010
Crassostrea madrasensis	Goa coast	Dry	Nr	17.9 – 65.1	6.4 – 86.4	Nr	Nagi, 2008
Saccostrea cucullata	Karnataka coast	Wet	1.5 – 201	Nr	0.3 – 7.5	1.47 – 10.9	Krishnakumar et al., 1998
Saccostrea cucullata	Deltaic Sundarbans	Dry	170 – 610	Bd	0	10 – 40	Sarkar et al., 1994
Saccostrea cucullata	Bombay coast	Dry	420	Nr	Nr	Nr	Krishnakumari et al., 1992
Saccostrea cucullata	Karwar coast	Wet	21.55 – 48.78	Nr	0.20 – 2.02	1.25 – 3.19	Krishna Kumar et al., 1990
Crassostrea madrasensis	Cochin coast	Dry	70 – 205	nr	nr	nr	Sankaranarayanan et al., 1978
Crassostrea gryphoides,	Goa coast	Dry	175 – 210	nr	nr	nr	Zingde et al., 1976
Saccostrea cucullata	Goa coast	Dry	251 – 728	nr	nr	nr	Zingde et al., 1976

Nr = Not reported, Bd = Below detectable limit

Statement of the Problem

Goa is rich in iron and manganese ore reserves in the hinterland that are mined from last few decades and contributing in state economy mainly by exporting to different countries (China, Japan, Taiwan, South Korea and Eastern European Countries) around the world (Goa exports 70.08 % of India's iron ore). The average annual iron ore production in Goa was about 28 million tonnes (Mt) during the years 2002–2011 and the mining waste generated during this period was thrice the amount of production (Sebastian et al., 2017). The Mandovi–Zuari estuarine system along with Cumbharjua canal is extensively being used for the transport of iron and manganese ores from hinterland to harbour. On the southern bank of Zuari estuary, near the mouth, Marmugoa harbour is situated which facilitates export of more than 10 million tonnes of iron ore annually (Dessai and Nayak 2009). The mining activities along with tourism, ship and barge building activities, fishing, discharge of waste from residential area keep the coastal waters of Goa under constant stress. Since harvesting of oysters is carried out from these areas, it is more likely that oysters from these areas may have higher concentration of metal because of their sedentary, filter feeding habitat. However, the knowledge of heavy metal concentrations in edible oyster species is very important with respect to human consumption of these species, nature management, and to determine the most useful biomonitor species and the most polluted area. In addition, biochemical and physiological responses in general and antioxidant defenses in particular is not well investigated in oysters from the region with reference to metal contaminants.

Oyster C. madrasensis and C. gryphoides were taken as Study Organism in the Proposed Work for Following Reasons:

- They are abundant in estuarine and coastal regions of Goa.
- They are filter feeder and have bioaccumulation capacity.
- Major beds of *Crassostrea* spp. are exposed to ship building and tourism activities.
- High edibility and economically important source of meat in Goa and neighboring states.

Objectives of the Study

Considering the demand and consumption of oysters, there is an urgent need to evaluate the status of metals concentrations in the oyster as well as in their ambience, and to understand antioxidant defense capacity of oyster *Crassostrea* species against accumulated metal. The detailed study on oyster from Goa coast was carried out with the help of field and experimental approach. The following objectives were set for the study:

1. Determination of spatial and temporal variation of heavy metal concentrations in *C. madrasensis*, *C. gryphoides* and surrounding environment (water and sediment)
2. Correlate the metal concentration in the tissue of *C. madrasensis* and *C. gryphoides* with physicochemical parameters of the ambient environment
3. Effect of heavy metals on *Crassostrea madrasensis* by using biochemical biomarker (Laboratory experiment)

Work Plan and Generic Structure of the Thesis

Literature survey

↓

Sampling at Zuari (Chicalim Bay), Mandovi (Nerul Creek) and Chapora (Chapora Bay) estuaries

(April 2013 – May 2014, field observations)

↓

Collection of *C. madrasesnsis*, *C. gryphoides*, and ambient surface seawater and surface sediment samples during low tide

↓

Analysis of environmental (hydrological and sedimentary) parameters (**Objective 2**)

↓

Estimation of metal (Cu, Ni, Pb and Cd) from whole oyster tissue, sediment, suspended particulate matter and seawater (dissolved) (**Objective 1**)

↓

Evaluation of antioxidant defense properties of *C. madrasensis* against dissolved Pb exposure (Experimental observations) (**Objective 3**)

↓

Interpretation of data obtained and preparation of scientific document (Thesis) for submission

The thesis is structured into six chapters.

Chapter 1 provides background information on the genus *Crassostrea* and the study species *C. madrasensis* and *C. gryphoides* in chronological order. It includes external morphology, anatomical features, reproduction and growth, food and feeding habits, ecological and economical importance of the oyster. In addition, antioxidant defense mechanism in general has been described.

Chapter 2 includes the detailed review of literature on oyster with respect to metal accumulation and ecotoxicological studies around the world and India. Moreover, the existing literature on oxidative stress and antioxidant defence properties against accumulated metals in oyster *Crassostrea* sp. have been given. Physico-chemical parameters and its variations in the estuarine and coastal waters of study area have also been included in this chapter.

Chapter 3 explains monthly variability in the climatic, hydrological and sedimentary properties of the ambient environment associated with oyster beds at the study sites. Results are presented in graphical and tabular forms.

Chapter 4 deals with monthly variations in heavy metals such as Copper (Cu), Nickel (Ni), Lead (Pb), and Cadmium (Cd) concentrations in the oyster tissue and surrounding matrices (surface sediment and particulate as well as dissolve form in surface seawater).

Chapter 5 deals with the antioxidant defense capacity of *C. madrasensis* against Pb stress.

Chapter 6 comprises the summary.

Chapter 3

Environmental Characteristics Influencing Oyster Beds Along the Goa Coast

Chapter 3

3.1 Introduction

Coastal ecosystems including estuaries are among the most valuable and yet highly vulnerable habitats in the world (Jickells, 1998). These ecosystems which have greater biodiversity than open ocean regions are increasingly becoming threatened due to growing population and exploitation pressure in most parts of the world (Blaber, 2000). The health of coastal ecosystems of India is being deteriorated due to the rapid introduction of pollutants of physical, chemical and biological origin resulting from various anthropogenic activities (Qasim and Kureishy, 1986). These pollutants act unfavorably to the aquatic organisms and change their biodiversity and alter the characteristics of the ecosystem (Hall and Ellis, 1985; House et al., 1993). Such environmental variables are greatly influencing the growth of the oyster and formation of the oyster beds. Therefore, detailed understanding of climatic, physical, chemical and biological environmental factors and their interaction from ambience is very essential for the survival and sustainable exploitation of fishery resources in general and molluscan fishery in particular.

The study area Goa is situated along the central west coast of India between 14°49’ and 15°42’ N and 73°38’ and 73°24’ E. Its extreme length from north to south is 105 km, breadth from east to west is 65 km, and its entire area is 3,702 km^2. It is bounded by the Terekhol River to the north which separates it from the state of Maharashtra. On the east side Western Ghats are present while Arabian Sea is present on western border of Goa. Southern and eastern region of Goa borders with Karnataka. Overall, it has ~105 km coastline intended with bays, creeks, promontories, sea cliffs, estuaries and world famous

beaches. Moreover, the state is intersected by nine rivers (Terekhol, Chapora, Baga, Mandovi, Zuari, Sal, Saleri, Talpona and Galgibag) which drains into the Arabian Sea. Out of these, two rivers (Mandovi and Zuari) are considered as lifeline of Goa because of their economic importance and area covered (69 % of the geographic area of the state). These rivers are used for ore transport, fishing, tourism activities, aquaculture, harbour development and waste disposal. Coastal areas also get pressurized by increasing human population which leads to habitat degradation, fragmentation and destruction (Gray, 1997). Such anthropogenic activities directly or indirectly may alter the physical, biological and geochemical conditions of the estuarine ecosystem to a considerable extent. Moreover, these estuaries are classified as a "monsoonal estuaries" (Shetye et al., 2007; Vijith et al., 2009), which receives abundant river discharge only during the monsoon and negligible discharge during the non-monsoon period. Several studies have been conducted on the physical, chemical and biological aspects of estuarine and coastal ecosystems of Goa region (Dehadrai and Bhargava, 1972; Goswami and Singbal, 1974; Singbal, 1976; Parulekar et al., 1980; De Sousa et al., 1981; Qasim and Sen Gupta, 1981; Ansari, 1988; Devassy and Goes, 1989; Rivonkar, 1991; Padmavati and Goswami, 1996; Nayak, 2002; Qasim, 2004; Rao et al., 2011; Kessarkar et al., 2013; Gauns et al., 2015). These studies have clearly demonstrated that hydrological parameters are highly influenced by the south-west monsoon and exhibits a rhythmic seasonal pattern.

Edible bivalves, particularly oyster *Crassostrea madrasensis, C. gryphoides*, *Saccostrea cucullata*, clam *Paphia malabarica*, *Meretrix casta*, and *Villorita cyprinoides* form

regular fishery are exploited round the year in Goa. They are confined to the shallow and intertidal regions of estuaries in close vicinity to or in regions influenced by different anthropogenic activities. Population density of oyster *Crassostrea* sp. along the Goa coast is reported in the range of 100–630 individuals/m^2 (Jagtap et al., 2011). Large oyster beds were most predominant in Chapora and Mandovi estuaries in North Goa and Zuari and Talpona estuaries in South Goa. Environmental conditions such as temperature, pH, salinity, dissolved oxygen, food availability, nutrient levels, and suspended load have considerable impact on the physiology of sedentary filter feeders such as oysters (Lenihan, 1999). Moreover, these ambient hydrological and sedimentary factors also plays a key role in regulating metal accumulation in the oysters (Depledge and Rainbow, 1990; Guo et al., 2001; Geffard et al., 2004). In recent years, rapid degradation of oyster bed and mortality due to pollution are observed. In view of oyster's economic importance as well as their vulnerability to the impact of urban and industrial developments, a survey of baseline is essential. Regularly monitoring of the ambient environment (surface water and sediment) of the oyster bed need to be initiated for its sustainable management.

Measures of reference physico-chemical parameters is often necessary to understand the estuarine health status. Due to complexity of relationship and interdependence of key environmental factors, it is difficult to draw a clear conclusion directly. However, statistical analysis can extract the underlying information and explain the structure of data in detail. In recent years, multivariate statistical techniques have been successfully applied to evaluate spatio-temporal variations of physico-chemical parameters caused by

natural and anthropogenic factors in coastal waters (Singh et al., 2004; Panda et al., 2006; Shirodkar et al., 2009; Wu et al., 2010; Sahu et al., 2013; Jha et al., 2014).

In this Chapter, multivariate statistical techniques have been used to explain the influence of possible sources (natural and anthropogenic) on water and sediment quality of Chicalim Bay, Nerul Creek and Chapora Bay from the Goa coast.

3.2 Materials and Methods

3.2.1 Selection of Sampling Sites

A survey of oyster beds was conducted along the coastal line of Goa for selection of sampling sites. It was found that oyster beds are under severe exploitation for human consumption and only empty shells were found in many places. Three sites along the Mandovi estuary, Zuari estuary and Chapora estuary having rich oyster beds were selected based upon the preliminary observations with respect to different anthropogenic ongoing activities and published literature on oyster beds from the state (Figure 3.1).

3.2.2 Sampling Sites

3.2.2.1 Chicalim Bay (CB)

Chicalim Bay (15°24’3.52” N, 73°51’14.24” E) is located on the southern bank towards confluence of Zuari estuary (Plate 3.1). This site hosts various shipbuilding industries, yards, workshops, and recreational anthropogenic activities. The site is exposed to iron ores transportations.

3.2.2.2 Nerul Creek (NC)

Nerul Creek (15º30’37.70” N, 73º46’48.75” E) opens into the Aguada Bay of Mandovi estuary (Plate 3.1). It extends inside the land in U-shape to a length of about 8.5 km. It is navigated by small fishing boats, and bounded by fringing and patchy mangrove vegetation. This site is in the influence of restaurants discharge (organic), fishing and other tourism activities. This site is also exposed to iron ores transportations from mines located upstream.

3.2.2.3 Chapora Bay (ChB)

Chapora Bay (15º36’30.43” N, 73º44’7.19” E) is also situated in the northern part of Goa (Plate 3.1) like NC. This bay is semicircular in nature and narrows down to join the Chapora River. Uniquely, this site is located far from main city. However, this site is exposed to major fish landing jetty, sewage disposal from land inhabitant along the bank of river.

3.2.3 Sample Collection

Oyster inhabits shallow intertidal or subtidal regions associated with sandy substratum, mixed with pebbles, gravels, dead shells and silt. The oyster beds get exposed during low tides, and hence samplings were carried out at ebb tide. Monthly sampling for a period of 14 months (April 2013–May 2014) was carried out at CB and NC. However, at ChB which was considered as a relatively pristine area, sampling was done once in four months covering three seasons viz. monsoon (July 2013), post-monsoon (November 2013) and pre-monsoon (March 2014). Surface water and sediment samples were

collected from the above mentioned sites and brought to the laboratory at low temperature in an ice box for hydrographic and geochemical analyses. All the samples were analyzed in triplicates.

3.2.4 Climatological Data Collection

The monthly average data of atmospheric temperature, solar radiation, wind speed, rainfall and humidity for study period were obtained from the Autonomous Weather Station (AWS) installed at the National Institute of Oceanography (NIO), Dona Paula, Goa, India.

3.2.5 Hydrological Parameters Analysis

Various hydrological parameters such as temperature, salinity, pH, dissolved oxygen (DO), nutrients [nitrate (NO_3-N), nitrite (NO_2-N) and phosphate (PO_4-P)], particulate organic carbon (POC) were analyzed by following standard oceanographic methods (Parsons et al., 1984).

3.2.5.1 Water Temperature

The surface water temperature was measured *in situ* with the help of a mercury thermometer graduated to 0.1 °C. The values are expressed in °C.

3.2.5.2 pH

Eutech Digital pH/EC pen (pH meter) was used to measure pH values in the water samples on the field.

3.2.5.3 Salinity

Salinity was determined by using hand refractometer (ATAGO, S/Milli—E).

3.2.5.4 Dissolved Oxygen (DO)

Dissolved oxygen concentrations were estimated by Winkler's method. This method involves fixation of dissolved oxygen using Winkler reagents A and B, immediately after collection of the 125 ml water sample in a dark bottle, followed by titration against standard sodium thiosulfate solution using starch as indicator. The results are expressed in mg/l of water.

3.2.5.5 Chlorophyll a (Chl a) and Phaeopigment

For estimation of total phytoplankton biomass (Chl *a*), 500 ml of surface water was filtered through the Whatman GF/F filter paper (47 mm diameter, 0.7 µm pore size) under low vacuum pressure. The pigments were extracted in 10 ml of 90 % acetone in the dark for 24 hrs at 4 °C. Fluorescence was measured for Chl *a* (before acidification with two drops of 1.2 N HCl) and for phaeopigments (after acidification) using Turner designs flurometer (10-AU) following the JGOFS protocol (UNESCO 1994). The Chl *a* and phaeopigment values are expressed as µg/l of seawater.

3.2.5.6 Nutrients

Nutrients were analyzed in the water samples after bringing it to room temperature. The samples were filtered through Whatman GF/F filter paper (47 mm diameter, 0.7 µm pore size) to remove high concentration of plankton and other suspended matter.

3.2.5.6.1 Nitrate-Nitrogen (NO_3-N)

NO_3-N was analyzed by reducing it quantitatively to nitrite by passing through a column containing cadmium filings coated with metallic copper (copper sulfate). Nitrite thus produced was then determined by diazotizing with sulfanilamide and coupling with N-(1-naphthyl) ethylenediamine to form highly colored azo dye, which was measured spectrophotometrically (Shimadzu UV mini-1240 model) at wave length 543 nm. Concentrations of NO_3-N are expressed in µmol/l.

3.2.5.6.2 Nitrite-Nitrogen (NO_2-N)

NO_2-N was measured by allowing the nitrite to react with sulfanilamide to form diazo compound, which reacts with N-(1-naphthyl) ethylenediamine and form highly colored azo dye, as described above in nitrate procedure. This dye was measured spectrophotometrically using wavelength of 543 nm. Nitrite concentrations are expressed in µmol/l.

3.2.5.6.3 Phosphate-Phosphorus (PO_4-P)

PO_4-P was measured by allowing the water to react with a composite reagent containing ammonium molybdate tetrahydrate solution, sulfuric acid, ascorbic acid and potassium

antimonyl-tartrate solution. The resulting complex was reduced to give a blue solution, which was measured spectrophotometrically at wavelength of 885 nm. Phosphate values are expressed in µmol/l.

3.2.5.7 Particulate Organic Carbon (POC)

Surface seawater (500 ml) was filtered through GF/F filter paper (47 mm, 0.7 µm pore size) and the filtrate was treated with 1 ml phosphoric acid and 1 ml distilled water. The mixture was heated in water bath (100–110 °C) for 30 minutes and allowed to cool at room temperature. Later 10 ml sulfuric acid-dichromate oxidant with 4 ml distilled water was added to the reaction mixture heated at 100–110 °C for 60 minutes and allowed to cool. The absorbance was measured spectrophotometrically at wavelength of 440 nm. The POC value were expressed in µg C/l.

3.2.5.8 Total Suspended Solids (TSS)

Surface water samples (500 ml) were filtered through a pre-weighed 0.45 µm Millipore membrane filter paper. The residue on the filters was dried at 60 °C till constant weight was obtained. The difference between initial and final weight of filter paper was recorded. Results of TSS (dry weight) were expressed in mg/L as described by Reddy et al. (1994).

3.2.6 Sedimentary Parameters Analysis

Sediment samples were dried in oven at 60 °C for 72 hours and stored in desiccators for further analyses for estimation of total organic carbon (TOC), total carbon (TC) and total nitrogen (TN).

3.2.6.1 Sediment Texture Analysis

Sediment samples were desalinated by washing repeatedly with deionized water and dried at 45 °C. Dried samples (15 g) were first disaggregated using 10 % hexametaphosphate and then treated with 30 % H_2O_2 for removal of organic matter. The sample was put through a 63 μm sieve (ASTM 230 mesh) to separate the sand fraction from the bulk sediment and was oven dried at 60 °C then weighed to calculate sand fraction percentage. The remaining mud (silt+clay) fraction from the same was analyzed by laser size particle analyzer (Malvern Mastersizer 2000). The data are presented as weight percentage (wt. %) in this study.

3.2.6.2 Sediment Elemental Analysis

For measurement of total organic carbon (TOC), sediment samples were decalcified by using 1N HCl, washed thoroughly with deionized water, and dried at 60 °C. The total carbon (TC) and the total nitrogen (TN) was analyzed without acid treatment. Then, concentration of TOC, TC and TN were measured using an elemental analyzer (Flash EA 1112 series, Thermo Fisher Scientific) and values are expressed in percentage.

3.2.7 Statistical Analysis

The basic statistics were applied on raw data set. The average ± standard deviation (SD), maximum and minimum values of surface water and sediment parameters were taken separately for each site using Microsoft Office Excel 2007. Pearson's correlation test was used to assess the significance of association between environmental parameters at each site. To determine the significance of spatial and temporal variation in water and sediment parameters, two-way analysis of variance (two-way ANOVA) was used. Further statistical analysis applied only on water parameters as the sediment parameters did not show significant variation in the two-way ANOVA analysis. Hereafter, values of water parameters at CB and NC were taken as an average of four months in each seasons: Mon (monsoon: June–September); PostM (post-monsoon: October–January) and PreM2 (pre-monsoon2: February–May, 2014 except PreM1 (: April–May, 2013). To minimize the effects of differences in measurement units and to deliver the data dimensionless all the water variables values were normalized. Cluster Analysis (CA) and Principle Component Analysis (PCA) were performed on normalized data. Based on CA, raw data were examined using Box–and–Whisker plots. All statistical computations were made using STATISTICA 8 (StatSoft, Inc., USA) and PRIMER 6 (Primer-E Ltd., Plymouth, UK) software (Clarke and Gorley, 2006).

3.3 Results

3.3.1 Climatic Parameters

3.3.1.1 Air Temperature

During the sampling period, the monthly average of atmospheric temperature ranged from 25.5 to 29 °C (Figure 3.2 a). Highest atmospheric temperature was 29 °C observed in May 2013, 2014 whereas, lowest temperature of 25.5 °C was recorded in the month of January.

3.3.1.2 Solar Radiation

Annual average of solar radiation during sampling period was 21.89 mW/sq.cm. Highest value (32.71 mW/sq.cm) was recorded during April, 2013 and lowest value (4.12 mW/sq.cm) was in February (Figure 3.2 b).

3.3.1.3 Wind Speed

The study area experienced heavy winds during monsoon season with peak value (2.91 m/s) in July whereas low wind speed (0.86 m/s) in November with annual average of 1.51 m/s (Figure 3.2 c).

3.3.1.4 Rainfall

During the study period, the first pulse of monsoon (pre-monsoon shower) was felt in May 2013. Further, monthly average of rainfall records for the months June-October showed heavy rainfall which tapered off in post-monsoon. Hence, the months of June, July, August and September are treated as the monsoon months. The total rainfall along

the Goa coast during the study period was about 7501 mm. (Figure 3.2 d). The pre- and post-monsoon constitute the dry season.

3.3.1.5 Humidity

The tropical climate of Goa is generally with humid conditions. The annual average of relative humidity reached up to 77.4 % which ranged from 63.48 % (December) to 91.61 % (July) (Figure 3.2 e).

3.3.2 Hydrological Parameters

3.3.2.1 Water Temperature

Temperature of overlying waters on oyster beds showed increasing trend from monsoon to pre-monsoon season (Figure 3.3 a). The highest monthly average temperature was 34 °C (April 2014), 32 °C (October) and 29.5 °C (March) at CB, NC and ChB, respectively, while lowest water temperature of 25-26 °C was measured in July (monsoon) at all the three sites.

3.3.2.2 pH

Low pH waters (range 6.2–6.7) over oyster bed were observed during the monsoon (July) at all the three sites (Figure 3.3 b). Highest pH values of 8.6 and 8.7 were noted at CB and NC respectively during September, while at ChB it was 8.1 in March. The annual average of pH during study period was 7.9, 7.7 and 7.4 at CB, NC and ChB, respectively.

3.3.2.3 Salinity

Salinity was found to be uniformly high with minute variation except during monsoon period (Figure 3.3 c). Lowest salinity value of 2 at CB and ChB, and 1 at NC was recorded during monsoon (July) season. While, highest salinity of 34 was recorded at CB and NC during May and February respectively. At ChB, maximum salinity (25) was observed in November 2013 and March 2014.

3.3.2.4 Dissolved Oxygen (DO)

Measurement of DO at CB ranged between 2.54 mg/l in April 2013 and 7.41 mg/l in July with an annual average value of 5.46 mg/l. At NC value of DO varied from 2.97 mg/l (May 2014) to 6.58 mg/l (February) while reference site (ChB) showed no significant variation with average value of 5.73 mg/l during seasonal observations. In general, DO concentrations showed inverse relation with temperature, pH and salinity (Figure 3.3 d).

3.3.2.5 Chlorophyll a (Chl a)

Chlorophyll *a* showed lowest concentration of 1.33 µg/l in July and highest value of 12.34 µg/l in June at CB with an annual average value of 7.34 µg/l. At NC, it varied from 2.02 µg/l (September) to 8.97 µg/l (May) with an average value of 4.77 µg/l. Measurement of Chl *a* at ChB did not show much variation which ranged between 2.27 and 3.87 µg/l during July and November respectively (Figure 3.3 e).

3.3.2.6 Phaeopigments

Annual average of phaeopigment showed highest concentration (9.85 µg/l) at CB compared to NC (2.43 µg/l) and ChB (1.71 µg/l). In detail, phaeopigment at CB varied from 3.76 µg/l (May 2013) to 34.0 µg/l (June), at NC, concentration ranged between 0.73 µg/l (August) and 5.82 µg/l (May 2013). Whereas, at ChB, values ranged from 0.63 µg/l (July) to 2.98 µg/l (November) (Figure 3.3 f).

3.3.2.7 Nutrients

Overall, during the monsoon season, dissolved inorganic nutrients (NO_3-N, NO_2-N and PO_4-P) of surface water were high, while with monsoon progression nutrient values decreased at all three study sites.

3.3.2.7.1 Nitrate-Nitrogen (NO_3-N)

NO_3-N showed highest monthly average values of 27.5 µmol/l, 24.48 µmol/l and 24.0 µmol/l at CB, NC and ChB, respectively in the month of July i.e. during monsoon period (Figure 3.3 g). This was followed by a gradual decrease from August. In contrast, the lowest concentrations of NO_3-N were noted at CB (0.94 µmol/l) in April 2014, at NC (0.01 µmol/l) in September and at ChB (0.45 µmol/l) in November.

3.3.2.7.2 Nitrite-Nitrogen (NO_2-N)

NO_2-N values ranged between 0.08 and 0.97 µmol/l in April and June, 2014, respectively at CB waters with an annual average value of 0.36 µmol/l. Likewise, NO_2-N concentrations with annual average value of 0.28 µmol/l showed similar ranges at NC

which varied from 0.10 µmol/l (October) to 1.04 µmol/l (July). At reference site ChB, annual average was relatively low (0.21 µmol/l) with values ranging between 0.11 µmol/l in November to 0.39 µmol/l in July month (Figure 3.3 h).

3.3.2.7.3 Phosphate-Phosphorus (PO_4-P)

PO_4-P concentrations showed minor fluctuations at all three sites except during monsoon season where highest peak was observed (Figure 3.3 i). The value of PO_4-P ranged between 0.43 µmol/l (December) and 9.18 µmol/l (June) in CB waters. However, at NC surface waters, it varied from 0.06 µmol/l to 3.96 µmol/l during January and August respectively. ChB showed PO_4-P variation within 0.21 µmol/l (November) to 3.57 µmol/l (July) range. Annual average of PO_4-P concentrations were 2.14 µmol/l, 1.14 µmol/l and 1.38 µmol/l at CB, NC and ChB, respectively.

3.3.2.8 Particulate Organic Carbon (POC)

Particulate organic carbon (POC) profile showed similar pattern at CB and NC waters (Figure 3.3 j). Maximum level of POC reached at CB (11325.6 µg C/l) and NC (6773.9 µg C/l) in September while minimum levels (2061 µg C/l at CB and 901.5 µg C/l at NC) were observed in month of November. Annual average of POC at CB and NC was 4349.3 µg C/l and 2224.6 µg C/l, respectively. POC concentrations at ChB were lowest 1137.4 µg C/l in month of March and highest 1824.1 µg C/l in July with slight variation (average value 1512.8 µg C/l).

3.3.2.9 Total Suspended Solids (TSS)

At CB, total suspended solid showed highest annual average concentration of 239.7 mg/l as compared to other two sites (NC: 29.9 mg/l; ChB: 17.4 mg/l). During monthly observation, total suspended solids ranged between 30.3–870.6 mg/l in April 2013 and June, respectively in CB waters. While at NC, suspended solids varied from 11.1 mg/l (February) to 86.1 mg/l (May, 2013). Quantity of suspended solids at ChB was much lower that varied within the range of 3.70 mg/l to 42.3 mg/l during March and July, respectively (Figure 3.3 k).

3.3.3 Sedimentary Parameters

3.3.3.1 Sediment Texture (Sand, Silt and Clay)

In texture analysis, sand fraction was dominated over the silt and clay fraction at all the studied sites. During monthly observations, sand content at CB varied from 57.3 % (December) to 97.0 % (September) with an average of 79.5 % (Figure 3.4 a), whereas at NC, sand content ranged between 66.2 % (September) and 90.1 % (May 2014) with an average of 80.1 % (Figure 3.4 b). In case of ChB site, sand content showed an average value 98.3 % with no significant variations (Figure 3.4 c).

Average percentage of silt at CB was 18.1 % which ranged from 2.4 % (September) to 37.9 % (December) (Figure 3.4 a). At NC, silt percentage varied from 8.9 % (May 2014) to 29.7 % (September) with an average value of 17.6 % (Figure 3.4 b). Samples from ChB showed very less silt content (an average 1.51 %) which varied between 0.80 % (July) and 2.49 % (March) (Figure 3.4 c).

The clay content at all three sites was low. At CB, NC and ChB an average of clay content was 2.34 %, 2.29 %, 0.14 % respectively. At CB, it ranged from 0.52 % in September to 5.35 % in March (Figure 3.4 a). Clay percentage at NC varied from 1.06 % (May 2014) to 4.08 % (September) (Figure 3.4 b). Whereas, at ChB clay values in July and November was 0.09 % and in March it was 0.25 % (Figure 3.4 c).

3.3.3.2 Sedimentary Element (TC, TOC and TN)

TC concentration at CB ranged between 0.17 % and 3.57 % in September and May, 2013 respectively. At NC, TC varied from 0.64 % (May, 2014) to 3.01 % (September). ChB showed variation of TC within 0.07 % (July) to 0.28 % (March). An average of TC concentrations was 1.39 %, 1.59 % and 0.18 % at CB, NC and ChB, respectively (Figure 3.5 a).

Similarly, the average TOC concentration at CB, NC and ChB was 0.85 %, 1.36 % and 0.11 % respectively (Figure 3.5 b). At CB, lowest TOC content (0.16 %) was observed in September and highest (2.05 %) in May 2013, whereas at NC, values ranged between 0.54 % (June) and 2.23 % (September). However, ChB showed much lower TOC values ranging between 0.08 % (July) and 0.13 % (March).

The TN concentrations were almost negligible at all the three sites (Figure 3.5 c). At NC, TN concentrations were 0.15 %, 0.18 % and 0.13 % in the month of May, September and

December, 2013 respectively. At CB, it was 0.12 % (March) whereas at ChB, it was below detection limit.

3.3.4 Statistical Analysis

Pearson's correlation test between physico-chemical parameters measured at study sites. At all the three study sites, the values of nutrients showed significant ($p<0.05$) negative relationship with temperature, salinity and pH. At CB, a significant ($p<0.05$) positive relationship was observed among POC, Chl *a* and phaeopigments (Table 3.1). The values of TSS showed a significant positive correlation with NO_3-N (r = 0.78, $p<0.001$), PO_4-P (r = 0.81, $p<0.001$), DO (r = 0.58, $p<0.05$) and phaeopigments (r = 0.63, $p<0.05$) whereas a negative relationship with salinity (r = –0.78, $p<0.001$). At NC, POC was negatively correlated with salinity (r = –0.54, $p<0.05$) (Table 3.2). Further, significant correlation was noticed between TOC and TC at CB (r = –0.92, $p<0.001$) as well as at NC (r = –0.93, $p<0.001$). At ChB, nutrients and TSS negatively correlated with salinity ($p<0.05$) (Table 3.3).

Two-way ANOVA was used to test the significant differences in temporal and spatial variation in hydrological and sediment parameters. The values of ANOVA demonstrated significant differences ($p<0.05$) among the sites, seasons and their interaction. Water parameters such as NO_3-N, POC and TSS showed a significant ($p<0.05$) seasonal and site wise variation (Table 3.4). However, a non-significant seasonal variation was observed among TOC, TC and TN of sediment (Table 3.5).

In cluster analysis, five distinct groups (Group A, Group B, Group C, Group D and Group E) were formed based on the seasonal variation in the studied sites (Figure 3.6). In Group A, only CB Mon was present While CB PreM1 and NC PreM1 clustered in another group (Group B). Site CB and NC showed similar characteristics in first pre-monsoon. Group C included NC PreM2, ChB PostM, ChB PreM, and NC PostM. This group showed more similarity between NC and ChB sites in pre- and post-monsoon season except during first pre-monsoon (PreM1) at NC. CB PreM2 and CB PostM formed another group (Group D). Overall, site CB formed a two separate clusters such as Group A and Group D in Monsoon and non-monsoon period respectively. Group E comprised of NC Mon and ChB Mon. Moreover, variables responsible for group formation in CA are explained by Box–and–Whisker plot (Figure 3.7). From the Box–and–Whisker plots, it is observed that most of the parameters are showing wide ranges of variation in Group A followed by Group E.

Results of PCA analysis are presented in Table 3.6 and Figure 3.8. Based on the Eigen value (>1), first three principal components (PCs) were retained as they explained 89.8 % of the total variability in the hydrological parameters. The PC1 explained 52.1 % of the variance and is associated with all nutrients (NO_3-N, NO_2-N and PO_4-P), POC and TSS which showed a negative loading with salinity. The second PC (PC2) described 28.5 % of the variability and characterized by positive loading of Chl *a*, pH and phaeopigments. The third PC (PC3) accounting for 9.2 % of the variance is mainly due to positive loading of DO.

3.4 Discussion

In the aquatic habitat, more particularly in the ecotone, the environment plays a paramount role in the life of the organisms. Ecosystem is a functional system which includes the organisms of natural community together with their environment (Dame, 1996). Any factor that disturbs the delicate balance between two components cause the chain of reactions and may end in drastic permanent changes resulting in restriction and reduction in species. Hence, evaluation of these changes by monitoring the environmental parameters has become an essential pre-requisite for the study of any organism. This is more relevant in the study of estuaries, bay and creeks as they are transitional zone with frequent fluctuating hydrological parameters. The water quality of aquatic bodies is not static but is subjected to change due to a number of factors, which are often natural or anthropogenic. Estuarine region of Goa represents unique hydrological features and are subjected to seasonal variations induced by the annual cycle of the monsoon.

Under hydrological observations, lowest temperature recorded during monsoon may be attributed to the speedy wind, heavy rainfall and the greater cloud cover which results in reduce solar radiation. Higher water temperature observed during pre-and post-monsoon could be due to the clear sky and associated higher atmospheric temperature. Similar observation was also recorded by earlier researchers (Qasim and Sen Gupta, 1981; Rivonkar, 1991). Lowest values of pH and salinity were observed during monsoon compared to non-monsoon period at all the three sites. Increased rainfall and dilution of seawater by the freashwater influx led to decreased salinity (1–2) of surface water. During monsoon heavy land runoff contribute large amount of organic material forming

organic acids due to decomposition, which may be one of the reasons responsible for decrease in pH at all the three sites. James and Najmuddin (1986) also observed a similar phenomenon in Palk Bay, Mandapam (India). The highest value of pH occurred during post-monsoon period have also been reported by Rivonkar (1991).

High value of DO occurred in monsoon season attributed to the increased solubility of oxygen in low saline condition of monsoon. Resultant could be due to the heavy precipitation and influx of oxygen rich freshwater from river runoff. High level of oxygen saturation has been reported earlier from estuarine water of Goa during monsoonal period (Singbal, 1976; Qasim and Sen Gupta, 1981). Higher value of pH and salinity coincides with low DO value in pre-monsoon season has also been observed by Ingole and Parulekar (1998). Furthermore, during monsoon POC values were high and could be associated with fresh water runoff and organic matter which release from substratum due to disturbance occurred in the monsoon (Jagtap, 1985). Variation in POC content attributed to the combination of factors such as variation in surface productivity (primary productivity) along with process that transfer organic matter over a period of time and secondly the advection of water masses having different POC content (Dehadrai and Bhargava, 1972). During monthly observations, relatively high concentrations of DO, Chl *a*, and phaeopigments were observed at CB than NC (Figure 3.3). Further, NO_3-N, NO_2-N, PO_4-P, POC and TSS were also found higher at site CB. This is possibly because of wider mouth of Zuari due to which more ingress and well mixing of sea water occurred compared to other sites. Site CB is also strongly influenced by shipping and harbour activities.

In sediment analysis, sand percentage was found to be higher whereas TOC and TC percentage were very low at ChB among the three sites (Figure 3.5). This low carbon content may be due to the dominant sandy nature of the sediment (Pradhan and Shirodkar, 2011). Comparatively high average value of TOC and TC of sediment were observed at NC, though percentage of clay is less compared to CB site. This may be attributed to mangrove vegetation at NC which contributes more input of organic material from the catchment area into sediment of the creek. Pradhan et al. (2014) also reported low values of TC and TN from Chapora estuary in comparison to Mandovi and Zuari estuaries. Furthermore, Shynu et al. (2015) found similar results of organic carbon (OC) (0.24–1.34 %) and TN (0.01–0.07 %) from Mandovi and Zuari estuaries.

Pearson's correlation test at the significant level of $p<0.05$ was applied to uncover the relationship among the ecological status indicators. At all the three study sites, the values of nutrients showed significant negative relationship with temperature, salinity and pH. This could be due to the terrestrial runoff containing nutrient rich domestic waste in the waters of studied sites. In contrast, at CB a significant positive relationship was observed among POC, Chl *a* and phaeopigments suggesting of productive nature of study site resulting in enhancement of the oyster population in this bay. However, this correlation increases the potential health hazard to human as oyster in this bay are exposed to various anthropogenic pollutants (metals, bacteria, etc.) that are assimilated along with food particles by filter feeders such as oysters in this bay. The observed values of TSS showed a significant positive correlation with NO_3-N, PO_4-P, DO and phaeopigments whereas a

negative relationship was observed with salinity (Table 3.1). This positive correlation indicates that the nutrient and oxygen rich freshwater carries suspended load into the estuary. At NC, POC was negatively correlated with salinity may be attributed to the particulate organic matter which enters the water body along with the freshwater efflux. In present study, statistically strong positive correlation between the fine particles (silt and clay), TOC and TN content observed at CB and NC sediments which may be due to high adsorption and affinity of organic carbon on finer particles in aquatic system. Additionally, a strong positive correlation between TOC and TN content was observed at NC (Table 3.2) which indicates that a major part of TN was associated with organic carbon as described by Gireeshkumar et al. (2013). However, a non-significant relationship was observed at ChB which may be attributed to the sandy nature of the sediment.

Cluster analysis was done using Euclidean distance test to detect the similarity in the water samples from three localities in four different seasons. The data analyzed in present study clearly shows that the monsoon clusters are widely separated from non-monsoon (Figure 3.6). The reason for the observed disparity between two seasons (monsoon and non-monsoon) could be due to the influence of south-west monsoon on the hydrology of studied water bodies. Further, Box–and–Whisker plots clearly presented the variation in variables could be due to higher influence of monsoon period (Figure 3.7). On other hand, variability in the concentrations of NO_3-N, NO_2-N, PO_4-P, POC and TSS are less in Group C which suggests that in non-monsoon season, NC and ChB site do not show much difference in environmental conditions. Presence of low DO values in PreM1 at CB

and NC water bodies was the main responsible factor to differentiate Group B from other groups. However, Group D which is characterized by higher values of phaeopigment, NO_3-N, NO_2-N, POC and TSS indicate poor water quality at CB in pre- and post-monsoon season. The characteristic values observed at CB could be due to commercial and industrial activities such as, fishing, ship building activities and iron ore transportation at this site. These activities enhance turbulence in the water which causes re-suspension of sediment. As a result, this phenomenon increases the phaeopigment levels in water, since the bottom sediments contain higher quantity of degrading pigments (Anand et al., 2014). The geomorphology of CB may be another reason for the observed values at this site where more sea water enters during daily tidal circulations at CB (Selvakumar et al., 1980). Mining rejects is also one of the major sources of NO_3 in the estuaries of Goa (De Souza, 1983).

The PCA approach was used to extract the important driving factors that are responsible for spatial and temporal variations in water quality. Further, comparison between loading and scoring on each PC have helped to identify the relationship between the variables and samples. The scoring on PC1 showed all the three sites during monsoon were influenced by high concentrations of nutrients (NO_3-N, NO_2-N, PO_4-P), POC and TSS (Table 3.6 and Figure 3.8). This indicates that all the studied sites (CB, NC and ChB) are affected by nutrient-rich freshwater runoff from land and resuspended fine-grained organic-rich sediments during the monsoon period. Similar results were obtained by other researchers when they evaluated the water quality of estuarine regions of Goa (Sardessai and Sundar, 2007; Anand et al., 2014). The PC2 explained that CB in all the seasons and NC during

PreM1 showed the highest values of Chl *a*, pH and phaeopigments. The third PC indicates high loading of DO which was observed at all the three sites during PostM period. Additionally, NC and ChB showed strong relationship with DO on PC3 in PreM2 and in PreM respectively. Variables Chl *a*, pH and DO showed positive correlation on PC2 and PC3 respectively mostly in pre- and post-monsoon. Generally, these variables are involved in various biological processes such as, photosynthesis, respiration activities and organic matter decomposition (Zang et al., 2011) which also influence the water quality. Similar results were observed in waters samples at CB throughout the year. From the PCA results, it is evident that in all the seasons, site CB is more influenced by land-based anthropogenic activities than other two sites (NC and ChB).

3.5 Conclusion

The different physico-chemical parameters measured in the present study have been used as an indicator for potential pollution in all the study sites (CB, NC and ChB). Based on the results of present study it is conclude that CB was most affected by anthropogenic activities while ChB was considerably pristine compared to the other two sampling sites during the study period. In recent years, increasing anthropogenic activities along the banks of Mandovi and Zuari rivers attracts the urgent need to monitor more parameters such as heavy metal concentrations in water, sediment and associated biota especially commercially important oyster species from these areas for the safety of human health and to create a comprehensive pollution database.

Table 3.1 Pearson correlation coefficient (r value) between physico-chemical parameters at CB (Chicalim Bay) during April 2013 – May 2014.

Parameters	Water Temp.	pH	Salinity	DO	Chl *a*	Phaeo.	NO_3-N	NO_2-N	PO_4-P	POC	TSS	Sand	Silt	Clay	TOC	TC	TN
Temp.	1																
pH	0.09	1															
Salinity	**0.63**[a]	0.25	1														
DO	-0.32	-0.24	-0.53	1													
Chl *a*	0.33	**0.64**[a]	-0.09	0.09	1												
Phaeo.	-0.11	0.31	-0.24	0.22	**0.59**[a]	1											
NO_3-N	**-0.72**[b]	-0.31	**-0.97**[c]	**0.54**[a]	-0.01	0.18	1										
NO_2-N	-0.24	0.27	-0.15	0.12	0.33	**0.76**[b]	0.23	1									
PO_4-P	-0.47	-0.17	**-0.71**[c]	0.26	0.22	**0.55**[a]	**0.74**[b]	**0.56**[a]	1								
POC	-0.37	**0.59**[a]	-0.34	0.12	**0.57**[a]	**0.68**[b]	0.28	0.43	0.27	1							
TSS	-0.48	-0.34	**-0.79**[c]	**0.59**[a]	0.18	**0.63**[a]	**0.78**[c]	0.51	**0.81**[c]	0.37	1						
Sand	0.06	0.21	-0.39	0.12	0.53	0.30	0.31	-0.07	0.31	0.53	0.28	1					
Silt	-0.06	-0.22	0.38	-0.13	**-0.54**[a]	-0.30	-0.30	0.08	-0.29	-0.53	-0.27	**-0.99**[c]	1				
Clay	-0.05	-0.13	0.38	-0.01	-0.44	-0.28	-0.33	0.01	-0.39	-0.48	-0.29	**-0.97**[c]	**0.96**[c]	1			
TOC	-0.06	-0.25	0.34	-0.17	-0.49	-0.33	-0.23	0.06	-0.19	-0.49	-0.24	**-0.87**[c]	**0.89**[c]	**0.77**[c]	1		
TC	-0.05	-0.46	0.25	-0.21	-0.53	-0.34	-0.13	-0.04	-0.07	-0.53	-0.13	**-0.73**[b]	**0.75**[b]	**0.59**[a]	**0.93**[c]	1	
TN	-0.05	0.01	0.14	0.01	0.01	-0.14	-0.19	-0.27	-0.18	-0.22	-0.19	-0.41	0.38	**0.56**[a]	0.26	0.19	1

Significance level at $p<0.05$ = a, $p<0.01$ = b, $p<0.001$ = c.

Abbreviations: Temp. = Temperature (°C), DO = dissolve oxygen (mg/l), Chl *a* = chlorophyll *a* (µg/l), Phaeo. = phaeopigment (µg/l), NO_3-N = nitrate (µmol/l), NO_2-N = nitrite (µmol/l), PO_4-P = phosphate (µmol/l), POC = particulate organic carbon (µg C/l), TSS = total suspended solids (mg/l), TOC = total organic carbon (%), TC = total carbon (%), TN = total nitrogen (%), sand, silt and clay (%).

Table 3.2 Pearson correlation coefficient (r value) between physico-chemical parameters at NC (Nerul Creek) during April 2013 – May 2014.

Parameters	Water Temp.	pH	Salinity	DO	Chl *a*	Phaeo.	NO_3-N	NO_2-N	PO_4-P	POC	TSS	Sand	Silt	Clay	TOC	TC	TN
Temp.	1																
pH	0.38	1															
Salinity	0.47	0.51	1														
DO	-0.28	0.19	-0.4	1													
Chl *a*	0.09	-0.51	-0.31	-0.51	1												
Phaeo.	0.42	0.06	0.18	-0.45	**0.59**[a]	1											
NO_3-N	-0.53	**-0.81**[c]	**-0.76**[b]	0.19	0.31	-0.15	1										
NO_2-N	**-0.58**[a]	**-0.58**[a]	-0.50	0.08	0.22	-0.05	**0.85**[c]	1									
PO_4-P	**-0.61**[a]	**-0.74**[b]	**-0.74**[b]	-0.01	0.49	-0.08	**0.73**[a]	0.51	1								
POC	-0.42	0.12	**-0.55**[a]	0.37	-0.15	-0.37	0.18	0.23	0.35	1							
TSS	0.23	-0.12	-0.14	-0.43	**0.74**[b]	**0.82**[c]	0.06	0.13	0.28	0.09	1						
Sand	-0.10	**-0.66**[a]	-0.17	-0.47	0.49	0.22	0.43	0.26	0.29	-0.45	0.16	1					
Silt	0.09	**0.64**[a]	0.17	0.45	-0.47	-0.21	-0.42	-0.23	-0.28	0.46	-0.14	**-0.99**[c]	1				
Clay	0.18	**0.71**[b]	0.14	**0.61**[a]	-0.53	-0.23	-0.47	-0.39	-0.36	0.37	-0.22	**-0.94**[c]	**0.92**[c]	1			
TOC	-0.41	0.21	-0.07	0.27	0.074	-0.10	-0.13	0.04	0.22	0.42	0.11	**-0.56**[a]	**0.57**[a]	0.52	1		
TC	-0.32	0.49	0.13	0.38	-0.23	-0.19	-0.36	-0.14	-0.05	0.44	-0.06	**-0.72**[b]	**0.72**[b]	**0.69**[b]	**0.94**[c]	1	
TN	-0.30	0.31	0.09	0.01	-0.02	0.28	-0.20	0.15	-0.01	0.44	0.43	-0.30	0.31	0.21	**0.54**[a]	**0.59**[a]	1.00

Significance level at $p<0.05$ = a, $p<0.01$ = b, $p<0.001$ = c.

Abbreviations: Temp. = Temperature (°C), DO = dissolve oxygen (mg/l), Chl *a* = chlorophyll *a* (µg/l), Phaeo. = phaeopigment (µg/l), NO_3-N = nitrate (µmol/l), NO_2-N = nitrite (µmol/l), PO_4-P = phosphate (µmol/l), POC = particulate organic carbon (µg C/l), TSS = total suspended solids (mg/l), TOC = total organic carbon (%), TC = total carbon (%), TN = total nitrogen (%), sand, silt and clay (%).

Table 3.3 Pearson correlation coefficient (r value) between physico-chemical parameters at ChB (Chapora Bay) during April 2013 – May 2014.

Parameters	Water Temp.	pH	Salinity	DO	Chl *a*	Phaeo.	NO_3-N	NO_2-N	PO_4-P	POC	TSS	Sand	Silt	Clay	TOC	TC
Temp.	1															
pH	0.97	1														
Salinity	0.96	**0.99**[a]	1													
DO	-0.98	-0.91	-0.89	1												
Chl *a*	0.73	0.87	0.89	-0.59	1											
Phaeo.	0.59	0.76	0.79	-0.43	0.98	1										
NO_3-N	-0.96	**-0.99**[a]	**-1.00**[b]	0.89	-0.89	-0.79	1									
NO_2-N	-0.95	-0.99	**-0.99**[a]	0.87	-0.91	-0.82	**0.99**[a]	1								
PO_4-P	-0.95	-0.99	**-0.99**[a]	0.87	-0.91	-0.82	**0.99**[a]	**1.00**[c]	1							
POC	-0.92	-0.80	-0.78	0.98	-0.40	-0.22	0.77	0.75	0.75	1						
TSS	-0.98	**-1.00**[b]	**-0.99**[a]	0.92	-0.86	-0.75	**0.99**[a]	0.99	0.99	0.81	1					
Sand	-0.86	-0.72	-0.69	0.94	-0.28	-0.09	0.68	0.66	0.66	0.99	0.73	1				
Silt	0.87	0.74	0.70	-0.95	0.30	0.12	-0.69	-0.67	-0.67	-0.99	-0.74	**-1.00**[b]	1			
Clay	0.74	0.56	0.52	-0.85	0.08	-0.11	-0.52	-0.49	-0.49	-0.94	-0.57	-0.98	0.97	1		
TOC	0.99	0.94	0.93	-0.99	0.65	0.49	-0.92	-0.91	-0.91	-0.96	-0.95	-0.91	0.92	0.81	1	
TC	0.98	0.90	0.88	**-1.00**[a]	0.57	0.41	-0.88	-0.86	-0.86	-0.98	-0.91	-0.95	0.96	0.86	0.99	1

Significance level at $p<0.05$ = a, $p<0.01$ = b, $p<0.001$ = c.
Abbreviations: Temp. = Temperature (°C), DO = dissolve oxygen (mg/l), Chl *a* = chlorophyll *a* (µg/l), Phaeo. = phaeopigment (µg/l), NO_3-N = nitrate (µmol/l), NO_2-N = nitrite (µmol/l), PO_4-P = phosphate (µmol/l), POC = particulate organic carbon (µg C/l), TSS = total suspended solids (mg/l), TOC = total organic carbon (%), TC = total carbon (%), sand, silt and clay (%).

Table 3.4 Two way ANOVA results (*p* value) of water parameters measured during April 2013–May 2014 with two factors (site and season).

Parameters	Factors	SS	Df	MS	F	*p*
Temp.	Site	7.30	1	7.30	3.21	0.077
	Season	127.86	2	63.93	28.08	*0.000*
	Site × season	8.44	5	1.69	0.74	0.594
pH	Site	0.54	1	0.54	2.44	0.122
	Season	6.95	2	3.48	15.56	*0.000*
	Site × season	5.43	5	1.09	4.87	*0.001*
Salinity	Site	64.53	1	64.53	2.04	0.157
	Season	5088.73	2	2544.37	80.47	*0.000*
	Site × season	129.03	5	25.81	0.82	0.542
DO	Site	4.43	1	4.43	6.66	*0.012*
	Season	9.06	2	4.53	6.81	*0.002*
	Site × season	1.91	5	0.38	0.58	0.719
Chl *a*	Site	51.19	1	51.19	5.54	*0.021*
	Season	0.11	2	0.05	0.01	0.994
	Site × season	171.10	5	34.22	3.70	*0.005*
Phaeo.	Site	764.30	1	764.30	24.05	*0.000*
	Season	81.76	2	40.88	1.29	0.282
	Site × season	578.19	5	115.64	3.64	*0.005*
NO_3-N	Site	111.28	1	111.28	4.32	*0.041*
	Season	3090.36	2	1545.18	60.04	*0.000*
	Site × season	455.48	5	91.10	3.54	*0.006*
NO_2-N	Site	0.06	1	0.06	1.48	0.228
	Season	0.83	2	0.42	11.18	*0.000*
	Site × season	0.26	5	0.05	1.41	0.229
PO_4-P	Site	29.16	1	29.16	0.54	0.463
	Season	413.82	2	206.91	3.85	*0.025*
	Site × season	82.24	5	16.45	0.31	0.908
POC	Site	77979241.76	1	77979241.76	35.39	*0.000*
	Season	62093671.49	2	31046835.74	14.09	*0.000*
	Site × season	29140402.36	5	5828080.47	2.65	*0.029*
TSS	Site	643480.80	1	643480.80	58.93	*0.000*
	Season	306767.87	2	153383.93	14.05	*0.000*
	Site × season	977575.61	5	195515.12	17.90	*0.000*

Italic value indicates significance level at $p<0.05$. Abbreviations: Temp. = Temperature (°C), DO = dissolve oxygen (mg/l), Chl *a* = chlorophyll *a* (µg/l), Phaeo. = phaeopigment (µg/l), NO_3-N = nitrate (µmol/l), NO_2-N = nitrite (µmol/l), PO_4-P = phosphate (µmol/l), POC = particulate organic carbon (µg C/l), TSS = total suspended solids (mg/l), Df = degrees of freedom, SS = sum of squares, MS = mean sum of squares, F = F value. Sites: Chicalim Bay, Nerul Creek, Chapora Bay. Seasons: pre-monsoon 1, monsoon, post-monsoon, pre-monsoon 2.

Table 3.5 Two way ANOVA results (*p* values) of sediment parameters measured during study period April 2013–May 2014 with two factors (site and season).

Parameters	Factors	SS	Df	MS	F	*p*
Sand	Site	48.09	1	48.09	0.82	0.368
	Season	445.35	2	222.68	3.79	*0.027*
	Site × season	1669.63	5	333.93	5.68	*0.000*
Silt	Site	38.81	1	38.81	0.86	0.357
	Season	331.16	2	165.58	3.67	*0.030*
	Site × season	1319.49	5	263.90	5.85	*0.000*
Clay	Site	0.29	1	0.29	0.29	0.593
	Season	7.41	2	3.71	3.68	*0.030*
	Site × season	18.79	5	3.76	3.73	*0.004*
TOC	Site	4.86	1	4.86	26.73	*0.000*
	Season	0.94	2	0.47	2.58	0.082
	Site × season	2.72	5	0.54	2.99	*0.016*
TC	Site	0.23	1	0.23	0.54	0.466
	Season	0.94	2	0.47	1.11	0.336
	Site × season	4.16	5	0.83	1.96	*0.094*
TN	Site	0.02	1	0.02	8.33	*0.005*
	Season	0.00	2	0.00	0.07	0.930
	Site × season	0.03	5	0.01	2.58	*0.032*

Italic value indicates significance level at $p<0.05$.
Abbreviations: TOC = total organic carbon (%), TC = total carbon (%), TN = total nitrogen (%), sand, silt and clay (%), Df = degrees of freedom, SS = sum of squares, MS = mean sum of squares, F = F value.
Sites: Chicalim Bay, Nerul Creek, Chapora Bay. Seasons: pre-monsoon 1, monsoon, post-monsoon, pre-monsoon 2.

Table 3.6 Principal component (PC) loadings and scores of water parameters at sampling sites CB (Chicalim Bay), NC (Nerul Creek), ChB (Chapora Bay) during April 2013–May 2014.

Variables	PC1	PC2	PC3	Samples	Score1	Score2	Score3
Temp.	0.31	0.29	0.11	CB PreM1	0.81	**0.35**	-1.75
pH	0.18	**0.44**	0.25	CB PreM2	1.15	**1.54**	**0.53**
Salinity	**0.33**	0.31	-0.21	NC PreM1	0.62	**0.40**	-2.01
DO	-0.21	-0.07	**0.84**	NC PreM2	2.12	-0.09	-0.32
Chl *a*	-0.14	**0.45**	-0.13	ChB PreM	2.02	-0.49	**0.87**
Phaeo.	-0.26	**0.42**	0.02	CB Mon	**-5.54**	**2.50**	0.27
NO_3-N	**-0.37**	-0.23	0.03	NC Mon	**-1.66**	-1.32	0.18
NO_2-N	**-0.36**	0.03	-0.20	ChB Mon	**-2.76**	-4.14	-0.02
PO_4-P	**-0.38**	-0.04	-0.34	CB PostM	-0.03	**1.75**	**0.56**
POC	**-0.34**	0.28	0.03	NC PostM	1.68	-0.09	**0.63**
SS	**-0.32**	0.33	0.07	ChB PostM	1.58	-0.42	**1.06**
Eigenvalues	**5.73**	**3.13**	**1.02**				
% Variation	**52.1**	**28.5**	**9.2**				
Cumulative %	**52.1**	**80.6**	**89.8**				

Abbreviations: Temp. = Temperature (°C), DO = dissolve oxygen (mg/l), Chl *a* = chlorophyll *a* (µg/l), Phaeo. = phaeopigment (µg/l), NO_3-N = nitrate (µmol/l), NO_2-N = nitrite (µmol/l), PO_4-P = phosphate (µmol/l), POC = particulate organic carbon (µg C/l), TSS = total suspended solids (mg/l), PreM1 = pre-monsoon 1, Mon = monsoon, PostM = post-monsoon, PreM2 = pre-monsoon 2.

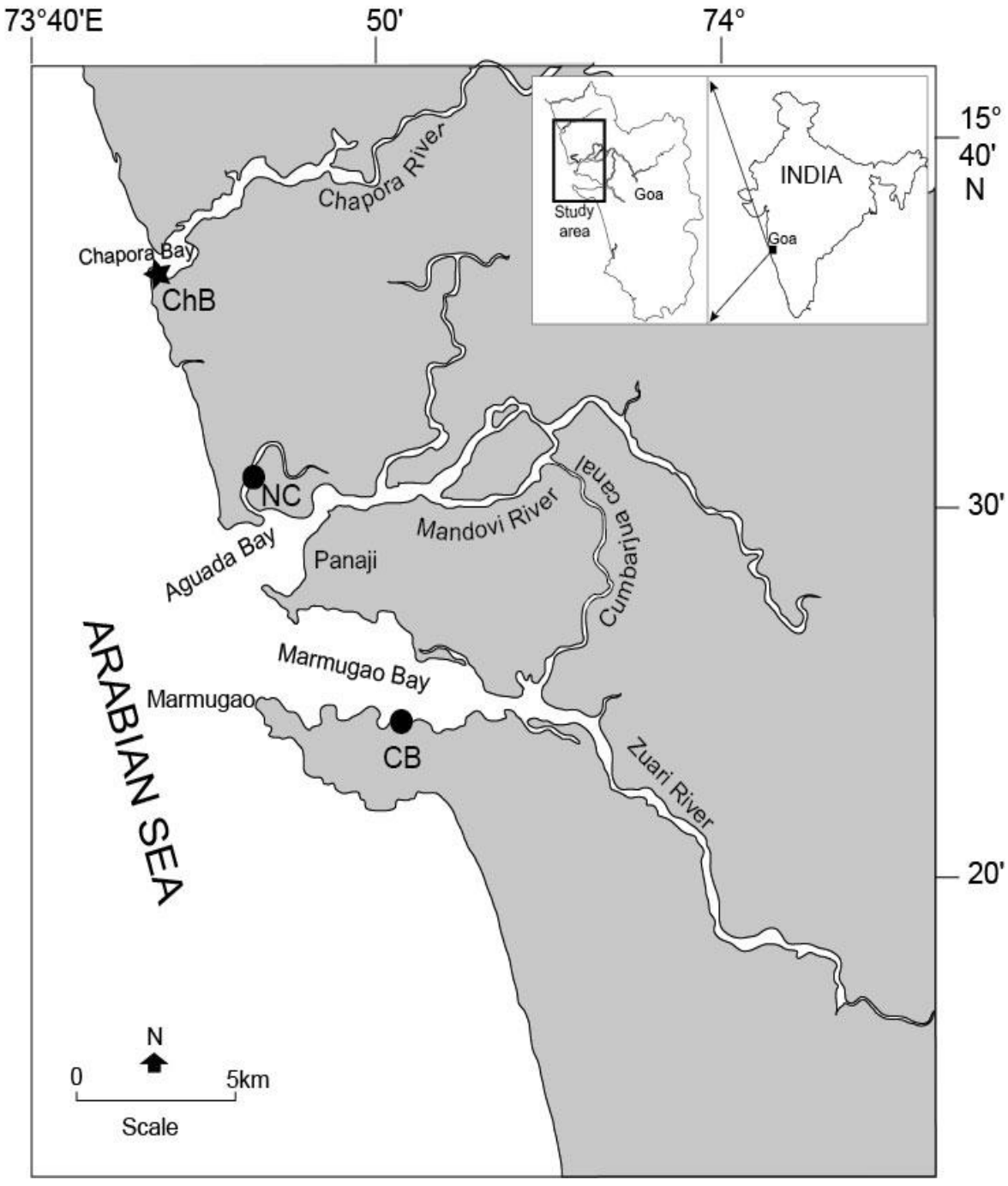

Figure 3.1 Map showing sampling sites (CB = Chicalim Bay in Zuari estuary, NC = Nerul Creek in Mandovi estuary, ChB = Chapora Bay in Chapora estuary) along the Goa coast (●: monthly sampling site and ★: seasonal sampling site).

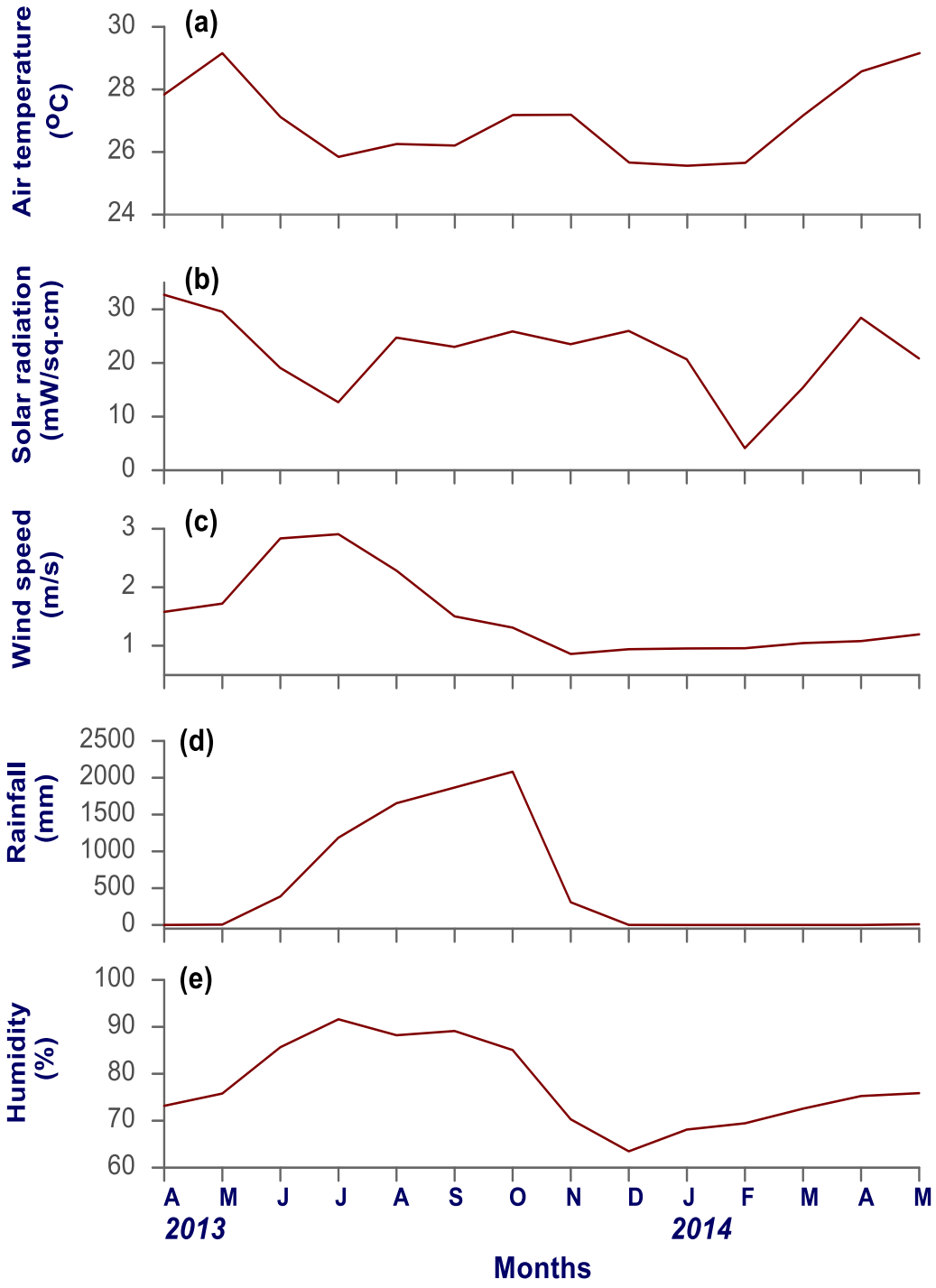

Figure 3.2 Monthly variations in (a) air temperature, (b) solar radiation, (c) wind speed, (d) rainfall and (e) humidity recorded along the Goa coast during April 2013 – May 2014.

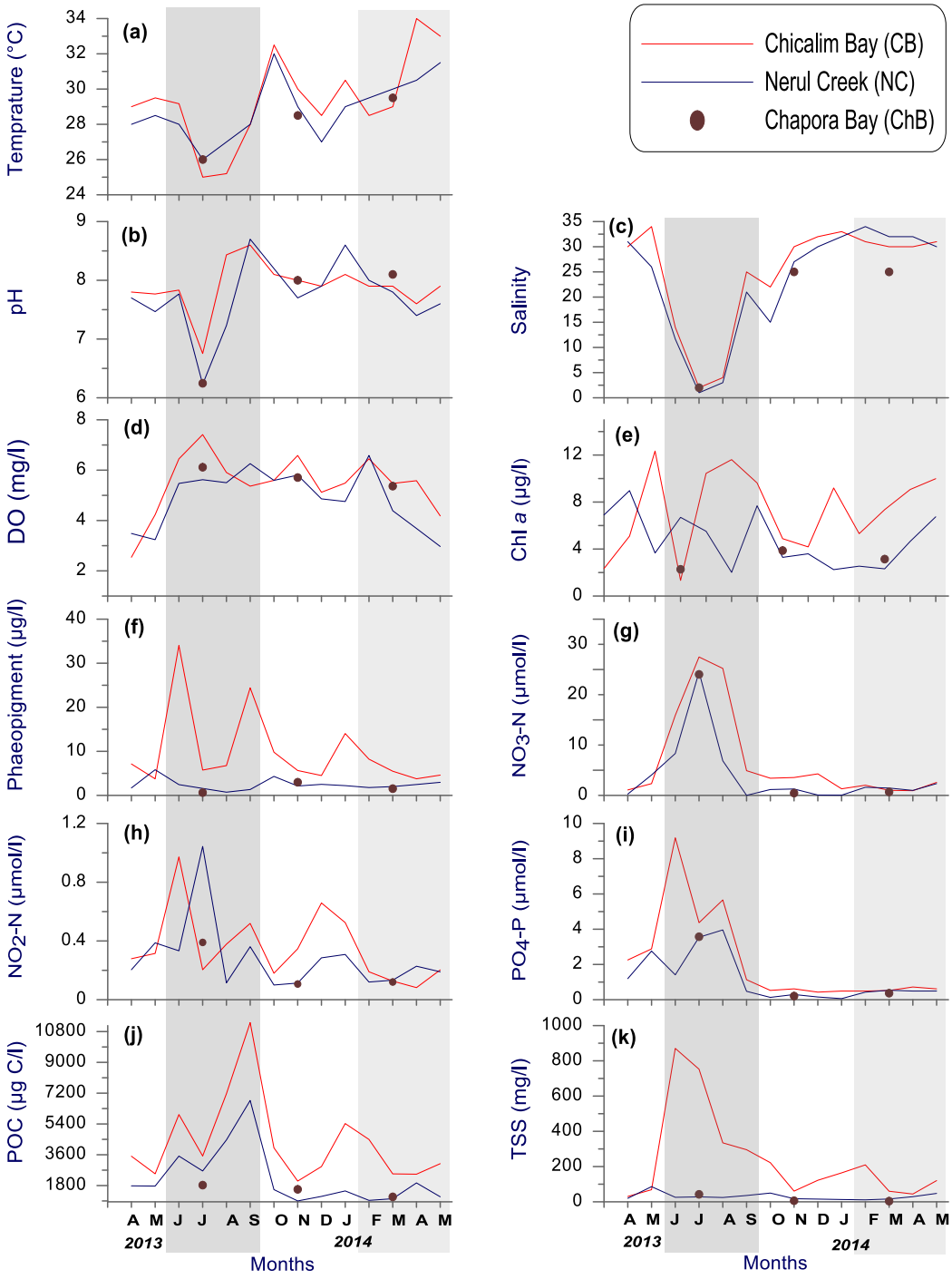

Figure 3.3 Monthly variations in (a) water temperature, (b) pH, (c) salinity, (d) DO = dissolve oxygen, (e) Chl *a* = chlorophyll *a*, (f) phaeopigment, (g), NO_3-N = nitrate, (h) NO_2-N = nitrite) (i) PO_4-P = phosphate, (j) POC = particulate organic carbon, and (k) TSS = total suspended solids recorded at sampling sites during April 2013 – May 2014. Values are presented as an average (n = 3).

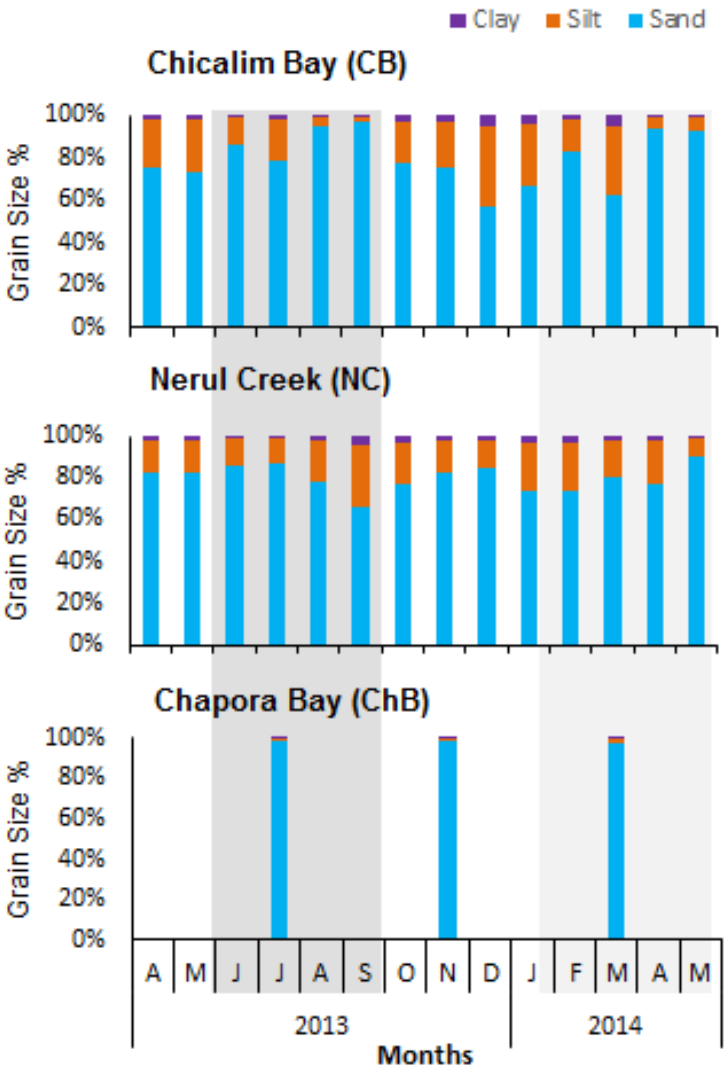

Figure 3.4 Monthly variations in sediment texture (sand, silt and clay) measured at (a) Chicalim Bay, (b) Nerul Creek, and (c) Chapora Bay during April 2013 – May 2014. Values are presented as an average (n = 3).

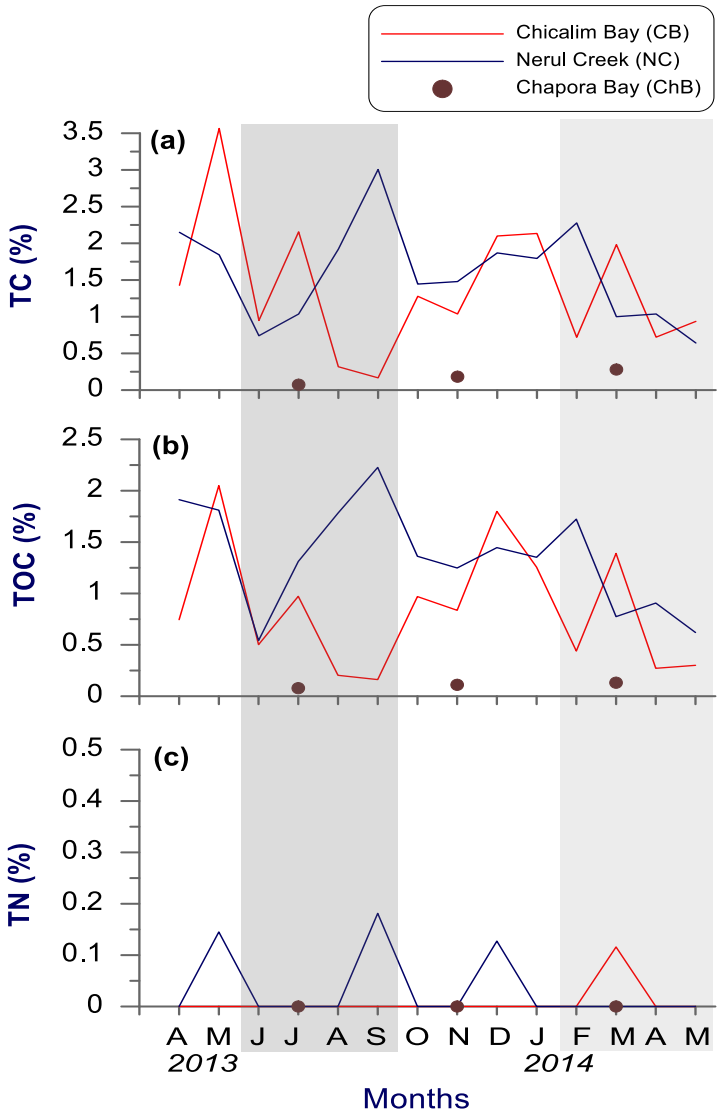

Figure 3.5 Monthly variations in (a) TC = total carbon), (b) TOC = total organic carbon), and (c) TN = total nitrogen measured at Chicalim Bay, Nerul Creek and Chapora Bay during April 2013 – May 2014. Values are presented as an average (n = 3).

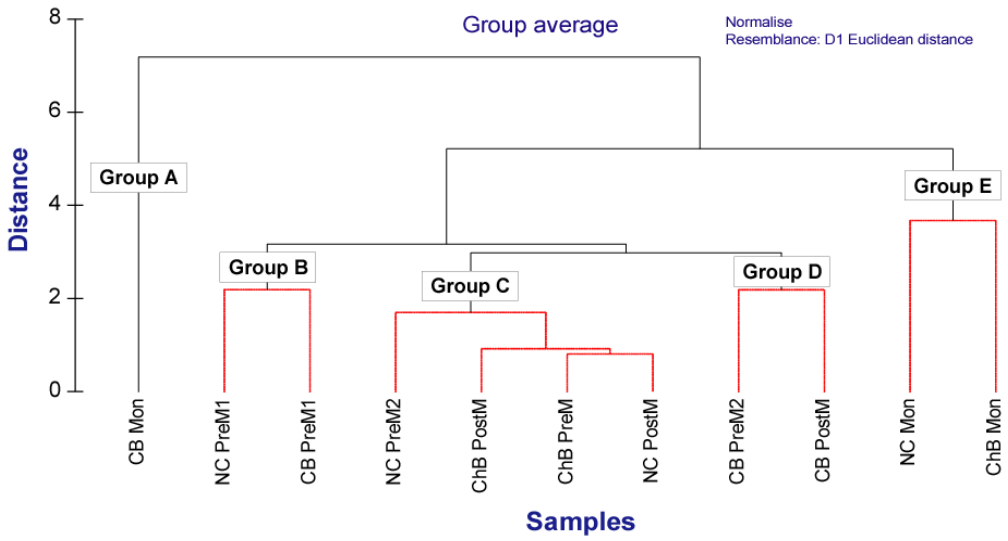

Figure 3.6 Cluster dendrogram based on hierarchical clustering method for seasonal variation in water samples at study sites. Abbreviation: CB = Chicalim Bay, NC = Nerul Creek, ChB = Chapora Bay, PreM1 = pre-monsoon 1, Mon = monsoon, PostM = post-monsoon, PreM2 = pre-monsoon 2.

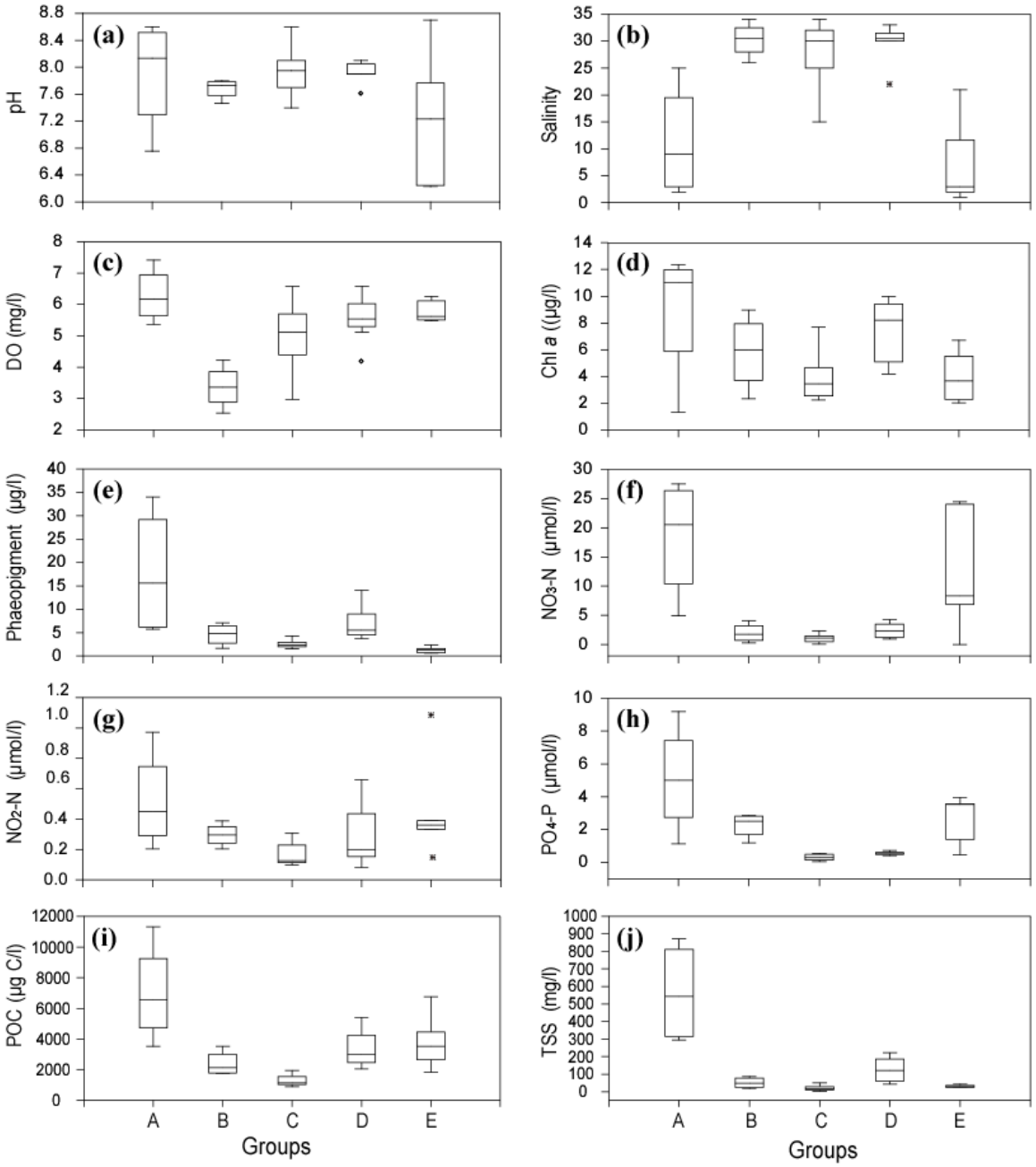

Figure 3.7 Box–Whisker–plot for (a) pH, (b) salinity, (c) DO = dissolved oxygen, (d) Chl *a* = chlorophyll *a*, (e) phaeopigment, (f) NO_3-N = nitrate, (g) NO_2-N = nitrite, (h) PO_4-P = phosphate, (i) POC = particulate organic carbon, and (j) TSS = total suspended solids in five groups formed in cluster diagram (Figure 3.6).

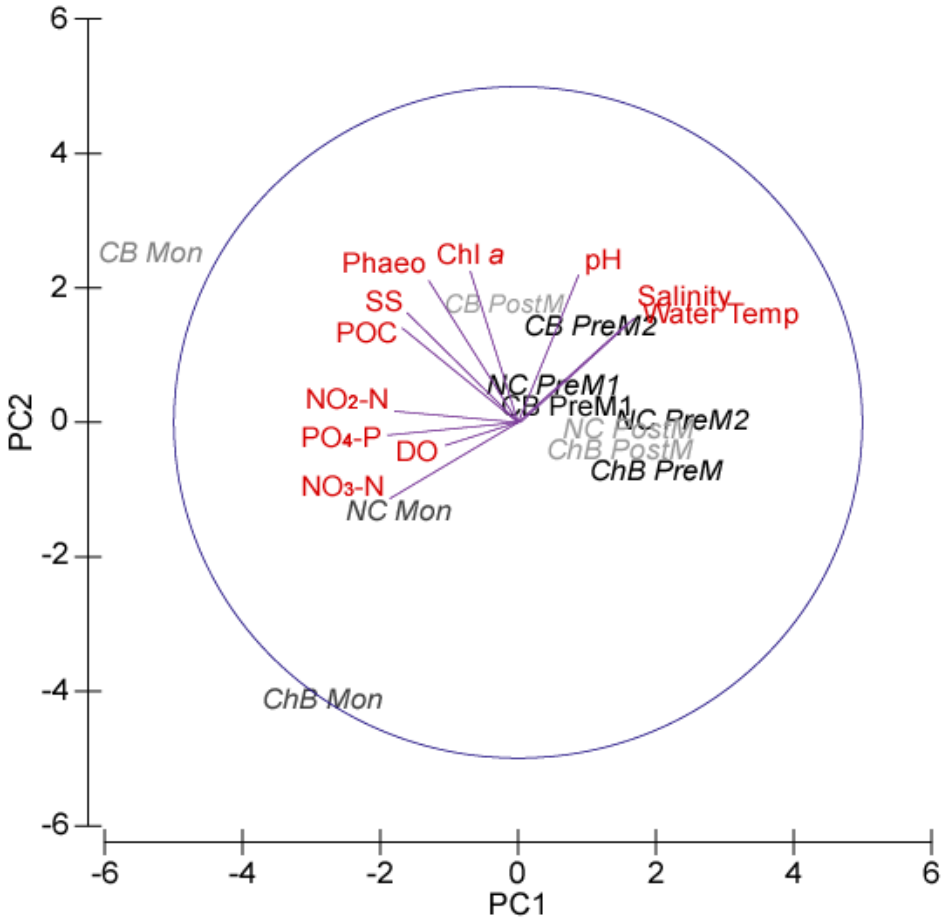

Figure 3.8 Principal component analysis diagram of water parameters measured at CB (Chicalim Bay), NC (Nerul Creek), and ChB (Chapora Bay) during April 2013 – May 2014. Abbreviations: Temp = Temperature (°C), DO = dissolve oxygen (mg/l), Chl *a* = chlorophyll *a* (µg/l), Phaeo = phaeopigment (µg/l), NO_3-N = nitrate (µmol/l), NO_2-N = nitrite (µmol/l), PO_4-P = phosphate (µmol/l), POC = particulate organic carbon (µg C/l), TSS = total suspended solids (mg/l), PreM1 = pre-monsoon 1, Mon = monsoon, PostM = post-monsoon, PreM2 = pre-monsoon 2.

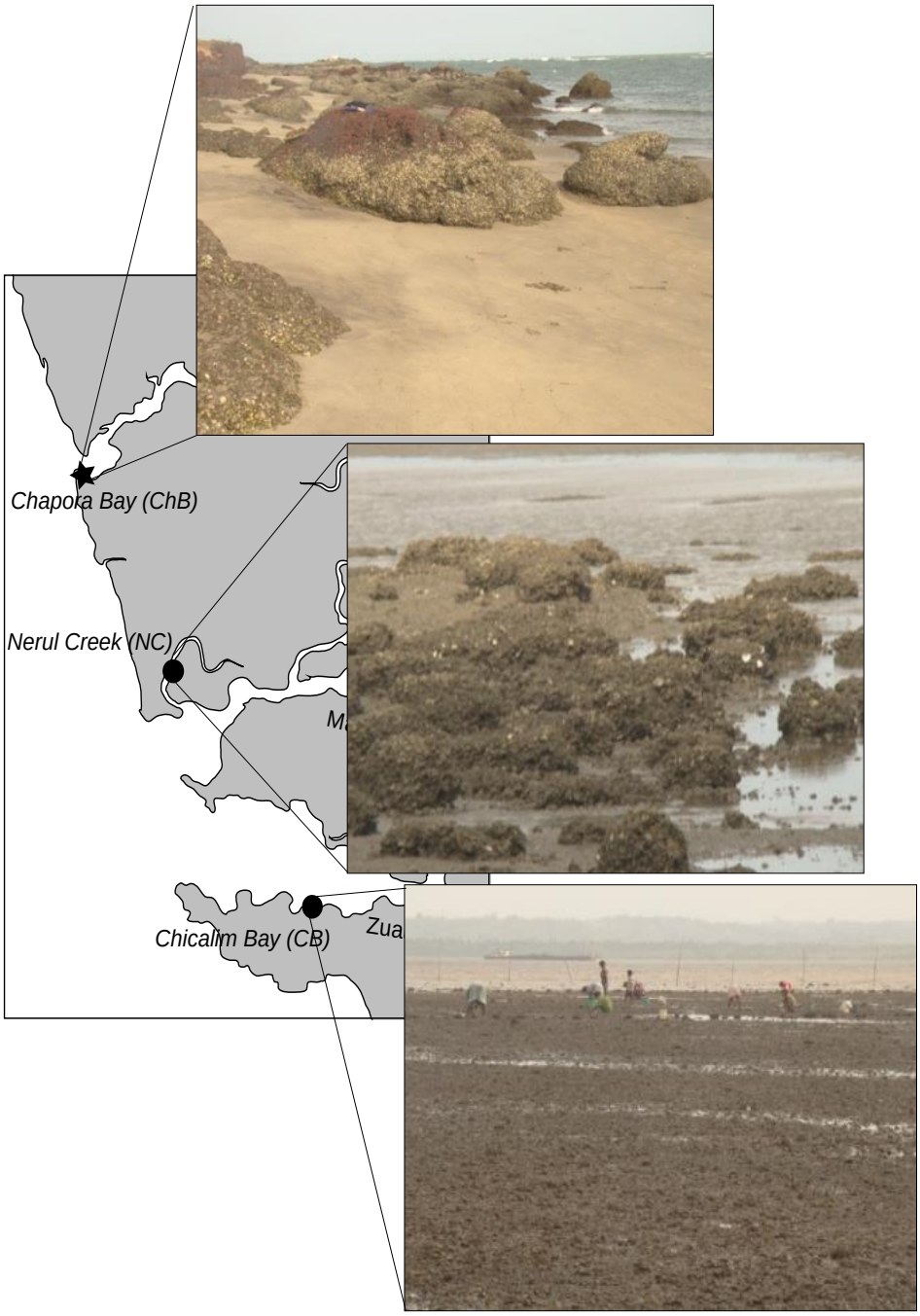

Plate 3.1 Photographs of sampling sites: Chicalim Bay in Zuari estuary, Nerul Creek in Mandovi estuary, Chapora Bay in Chapora estuary along the Goa coast (●: monthly sampling site and★: seasonal sampling site).

Chapter 4

Status of Heavy Metals Concentration in Oysters and Ambient Environment Along the Goa Coast

4.1 Introduction

Heavy metals (also referred as trace metals) are considered as highly toxic pollutants due to their toxicity, persistence and bioaccumulation properties (Pekey, 2006). These metals contaminants when released into the sea, significantly affect marine ecology, and in extreme cases, lead to the destruction of whole ecosystems including sediment and, water quality and living biota. Coastal and estuarine habitats (between land and sea) serve as a sink for heavy metals and thus, these regions remain constantly under pressure for contaminations.

Marine environmental components such as sediments act as a final reservoir for metals (with particulate forms, various chemical species, and different complexes) with their deep effect on bioavailability and toxicity to marine organisms (Elderfield and Hepworth, 1975; Nelson and Donkin, 1985). Aqueous (particulate and dissolve) phase provide a mobile medium for metal chemical reactions, metal circulation, through the sediment to organism and to aquatic environment (Violante et al., 2010). It is well known that oysters significantly accumulate heavy metals from seawater from three sources: inorganic metal ions, organometallic ion complexes and metal ions accumulated by phytoplankton.

Sediment resuspension is one of the natural processes that entrains sinking particles back into the upper water column and this particulate matter has a considerable geochemical importance. Suspended particulate matter (SPM) acts as a carrier phase to transport the chemical elements from seawater to the bottom sediments and vice versa. Its production, sinking and decomposition control the recycling and distribution of elements in marine waters to a large extent. Therefore, establishing a correlation of metal levels in sediment, water column and uptake in biota is

essential to understand the influence of environmental metal contaminants on the food chain in estuarine environment. Further, in order to differentiate and understand the mechanisms that control bioaccumulation in marine organisms, its uptake and pathway must be separately quantified and all of the geochemical, biological, and physico-chemical conditions that a population of animals may experience need to be considered (Wang and Fisher, 1999).

Sedentary bivalves are directly and continuously exposed to marine contaminants. Oyster lives near the sediment-water interface and hence get influenced by heavy metals in sediment. Bivalves, especially the oysters, have been widely used to assess the metal pollution due to their ability to accumulate metals from contaminated marine environments (Hunter et al., 1995; O'Connor, 2001). Consequently, oysters from contaminated sites serve a potential risk to human health. Metals such as lead (Pb), cadmium (Cd) and mercury (Hg) have been given special attention in environmental monitoring studies especially in biological samples because bioaccumulation of these metals can cause potentially toxic effects to the organism and to humans who consume it as a seafood (Bragigand et al., 2004; Amiard et al., 2008).

India is fortunate to have large resources of oysters with an annual production of about 24,000 tonnes (http://www.fao.org/fishery/countrysector/naso_india/en). However, edible oysters such as *Crassostrea madrasensis* and *C. gryphoides* are under severe fishing pressure including the Goa coast. Despite its importance as a major natural fisheries resource in Goa, oyster beds are exposed to increasing pressures of different anthropogenic activities mainly related to urban development, ship building and mining activities in the estuarine regions. There are several reports on metal contamination in Mandovi and Zuari estuarine waters (Alagarsamy, 2006;

Fernandes and Nayak, 2009; Kessarkar et al., 2013; Shynu et al., 2013; Veerasingam et al., 2015). But very few studies have been conducted on metal accumulation in marine organisms (Krishna kumari et al., 2006; Bhat et al., 2014), and no such data is available for edible oysters along the Goa coast.

Therefore, considering the demand and local consumption of oyster, there is an urgent need to evaluate the status of metal accumulation in commercially important oyster *Crassostrea* sp. that are harvested regularly from Goa coast for human consumption. Keeping this aspect in forefront this chapter deals with the following objectives (1) to estimate bioaccumulation of copper (Cu), nickel (Ni), lead (Pb) and cadmium (Cd) in the tissue of two oyster species (*C. madrasensis* and *C. gryphoides*) and to assess whether the oysters could be used for human consumption from selected sites of Goa coast (2) to evaluate the current status of metal concentrations in surrounding matrices (surface sediment, SPM, and surface seawater) of the oysters (3) to understand relationship, if any, exist between the accumulated metal in oyster tissue and in total metal content in surrounding mediums and other environmental controlling factors.

4.2 Materials and Methods

4.2.1 Study Sites

Detail information regarding the study sites has been mentioned in Chapter 3 Section 3.2.2 and refer Figure 3.1.

4.2.2 Collection and Processing of Samples

4.2.2.1 Collection of Samples

Surface (upper 2 cm) sediment (~100 g) samples was scraped using a stainless steel spoon and collected in metal-free polyethylene bags. Surface seawater (~ 5 l) was collected in polyethylene jerry cans. Before sample collections, cans were washed with detergent, soaked in 5 % nitric acid (HNO_3) for overnight, and then washed with distilled water. Seawater samples were taken carefully to avoid the floating debris. Suspended particulate matter was collected by filtering the collected seawater through acid treated 0.4 µm polycarbonate membrane filter paper. The SPM retained on filter papers was used for further analysis. Filtered surface seawater samples (400 ml) were collected in acid washed polyethylene bottles and pH was adjusted upto 2.5 using HNO_3.

To avoid the size difference effect on analysis, only 6–7 cm length of oysters were chosen during collection. In the laboratory, oysters were cleaned with sea water to remove adhering sediment. Subsequently, oysters were shucked and whole soft tissue was cleaned with deionized water to remove impurities. Oysters were identified to the lowest taxonomic level following the method described by Durve (1967, 1974) and Siddique and Ahmed (2002). All the samples were well packed in clean container and kept on ice until brought back to the laboratory where the sediment and water samples were stored at 4 °C and the oyster kept at -20 °C.

4.2.2.2 Total Metal Determination in Surface Sediment

Sediment samples were dried in oven at 60 °C for 72 hours and pulverized using agate mortar and pestle. Next, 0.05 g sediment powder of each sample was taken in Teflon beaker and 10 ml of $HF:HNO_3:HClO_4$ (7:3:1 ratio) acid mixture was added and kept for overnight soaking for

reaction. Next day, mixture was heated at 180 °C until dryness. Dryness step was repeated in 5 ml of above mentioned acid mixture. Digested samples were cooled at room temperature and then diluted to 10 ml with 2 % HNO_3. Subsequently, 5 ml of 1 mg/kg Rh solution was added as an internal standard and made up to the final volume. Marine sediment, MAG-1 used as certified reference material to check the data quality. Quantification of metals was done by using Thermo X Series II Inductively-Coupled Plasma Mass Spectrometer (ICP-MS).

4.2.2.3 Total Metal Determination in suspended particulate matter (SPM)

Suspended particulate matter containing filter paper was dried at 60 °C and weighed. Next, 0.05 g of SPM was taken and analyzed as described in above 4.2.2.2 section.

4.2.2.4 Dissolved (< 0.4 μm) *Metal Determination in Surface Seawater*

The dissolved metal content in seawater was determined following the method of Brewer et al. (1969). Metals from filtered water samples (400 ml) were pre-concentrated from seawater using 10 ml ammonium pyrolidine dithiocarbamate (APDC) as chelating agent and in 15 ml methyl isobutyl ketone (MIBK) as solvent. Repetition of the above process was done for leftover seawater using 5 ml of APDC and 10 ml of MIBK and the upper organic layer was decanted. The organic extract was further treated with 0.2 ml HNO_3. After that inorganic form of study metals were aspirated in Graphite Furnace Atomic Absorption Spectrometer (GF-AAS).

4.2.2.5 Total Metal Determination in Oyster Tissue

Sample of each oyster species (*C. madrasensis* and *C. gryphoides*) consisted a pool of 20 oysters. Whole tissue of oysters was dried at 60 °C till constant weight. The dried tissue samples were

grinded by using agate mortar and pestle. The 0.5 g of dried tissue powder was soaked overnight in 5 ml of 65 % HNO_3. Reaction mixture was heated at 80 °C till dryness and re-dissolved in 3 ml of 65 % HNO_3 for achieving the complete digestion of the tissue. The digest was allowed to cool at room temperature. The digested clear solution was then filtered through Whatman No. 42 filter paper and diluted to 10 ml with 2% HNO_3 used for metal determination (Cheung and Wong, 1992). Fish protein certified reference material (DORM–4) was used to ensure the quality of the results. Metal (Cu, Ni, Pb, and Cd) determination in tissue of both the oyster species were made by Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES).

Utmost care was taken at every step in sample processing to avoid contamination. All reagents used in the analysis were suprapure grade. Appropriate dilutions of samples were made depending on the sensitivity of detection in these samples. Appropriate blanks and standards were also prepared by using the same method. Precision measured as percent relative standard deviation (% RSD = standard deviation / average × 100) of triplicate values were <10 %. Accuracy of Cu, Ni, Pb and Cd analyses are expressed in recovery percentage (Recovery % = (Measured value of standard / certified value of the standard) × 100) (Table 4.1).

4.2.3 Statistical Analysis

The basic statistics such as the average ± standard deviation (SD), were calculated for all measured heavy metals concentrations using Microsoft Office Excel 2007. Pearson correlation test (r values) was performed on untransformed data to find out the degree of association between the metal concentrations in oyster tissue, metal content in their ambience (sediment,

seawater (particulate and dissolve phase)) and the other measured physico-chemical (refer Chapter 3) parameters using STATISTICA 8 (StatSoft Inc., USA) software.

Further statistical analysis were carried out on 4 seasons (an average of 4 months were grouped in each season) such as Mon (monsoon: June–September 2013), PostM (post-monsoon: October 2013–January 2014), PreM2 (pre-monsoon 2: February–May 2014) except PreM1 (pre-monsoon 1: April–May 2013) at Chicalim Bay (CB) and Nerul Creek (NC). In case of Chapora Bay (ChB) statistical test was performed on three season representative months: monsoon (July 2013), post-monsoon (November 2013), and pre-monsoon (March 2014). All assumptions of normality and homogeneity of variance were tested using Kolmogorov–Smirnov test and Levene's test respectively. However, when data failed to meet normality, permutational multivariate analysis of variance (PERMANOVA) test was used on metal values in sediment, particulate and dissolved phase in seawater. PERMANOVA was performed on untransformed data to find out the significance level in spatio-temporal variations in the samples using PRIMER 6 (Primer-E Ltd., Plymouth, UK) software (Clarke and Gorley, 2006).

To perform Redundancy Analysis (RDA), measured physicochemical variables (such as water temperature, pH, salinity, DO, Chl *a*, NO_3+NO_2, PO_4, POC, TSS, sand, silt, clay, TOC, TC) (refer Chapter 3) and metals concentration in sediment, in seawater (dissolved and particulate form) and in oyster tissue were retained. To reduce the overcrowding of the environmental variables in ordination diagram, variables were divided into three different groups. First, water parameters (water temperature, pH, salinity, DO, Chl *a*, NO_3+NO_2, PO_4, POC, TSS); second, sediment parameters (sand, silt, clay, TOC, TC), sediment metals (Cu, Ni, Pb, Cd); and third,

metal (Cu, Ni, Pb, Cd) content in particulate and dissolve phase. To determine the variability within each of these data sets Detrended Correspondence Analysis (DCA) was performed in order to find out the length of gradient of the dataset. The longest gradient was found to be less than 4.0 indicating a linear distribution of metal accumulated oysters and justifying the use of a linear method, RDA for further test (Leps and Smilauer, 2003). Monte Carlo permutation test was performed to determine whether the relation between biological and environmental variables was significant. For all the analyses significant level $p<0.05$ were considered. For this multivariate analysis, CANOCO 4.5 for windows (ter Braak and Smilauer, 2002) software was used.

4.3 Results

The trend of metals accumulation in the tissue of *C. madrasensis* and *C. gryphoides* was in the following orders: Cu > Cd > Ni > Pb. The observed metals concentration in the sediment (Cu > Ni > Pb > Cd) and in the SPM (Ni > Cu > Pb > Cd) was different from that of concentration in tissues at CB and NC. But at ChB sediment and SPM, it showed vice-a-versa trend. Further, dissolve phase of metals were in the following ranking: Cu > Pb > Ni > Cd at CB, Cu > Ni > Pb > Cd at NC and Cu > Ni > Cd > Pb at ChB.

4.3.1 Concentration of Cu

4.3.1.1 Surface Sediment

Cu content at CB sediment varied from 55.3 mg/kg (January) to 1182 mg/kg (September) whereas at NC and ChB, concentrations occurred in the ranges of ~31 mg/kg (November 2013 and May 2014) to 76.2 mg/kg (May 2013) (Figure 4.1). It is important to point out that the

average value of Cu content (211.8 mg/kg) in CB sediment was much higher than sediment from NC (47.9 mg/kg) and ChB (54.5 mg/kg). Moreover, Cu enrichment in sediment from all the three study sites (CB, NC and ChB) were observed in monsoon period.

4.3.1.2 Suspended Particulate Matter (SPM)

At NC and ChB, SPM Cu level was found more as compared to sediment Cu. Average Cu content was 105.1 mg/kg, 76.7 mg/kg and 146.5 mg/kg at CB, NC and ChB, respectively. Highest particulate Cu was observed at ChB (range: 100.4 – 197.4 mg/kg) followed by CB (range: 77.9 – 129.2 mg/kg) and NC (range: 62.2 – 94.7 mg/kg). At CB, seasonal average of particulate Cu content depicted equally high level in all the seasons (except in pre-monsoon 1) whereas at NC and ChB waters, particulate Cu level was more in monsoon and less in pre-monsoon season (Figure 4.1).

4.3.1.3 Seawater (Dissolved)

Average dissolved Cu content in seawater at CB, NC and ChB was 2.34 µg/l, 2.21 µg/l and 1.85 µg/l, respectively. Monthly observations of dissolved Cu ions varied between 1.31 µg/l (August) and 4.30 µg/l (November) at CB, 0.85 µg/l (October) and 4.59 µg/l (November) at NC, 1.44 µg/l (March) and 2.37 µg/l (November) at ChB. Overall, it was noticed that dissolved Cu level at CB was high in monsoon and at NC and ChB in non-monsoon season (Figure 4.1).

4.3.1.4 Oyster Tissue

Concentrations of Cu in tissue of both the oysters (*C. madrasensis* and *C. gryphoides*) at all the sites were in similar ranges. In particular, average Cu content in *C. madrasensis* at CB, NC and

ChB was 408.4 mg/kg, 398.1 mg/kg and 329.9 mg/kg, respectively. In case of *C. gryphoides* tissue, it was 316.7 mg/kg, 284.6 mg/kg and 526.5 mg/kg, respectively (Figure 4.1). Seasonal average showed comparatively more Cu content in oyster tissues collected from CB and ChB during monsoon whereas at NC, concentration of Cu was higher during pre-monsoon 2.

4.3.2 Concentration of Ni

4.3.2.1 Surface Sediment

Average Ni content in surface sediment at CB, NC and ChB was 53.4 mg/kg, 42.6 mg/kg and 57.7 mg/kg, respectively. Monthly observations at CB varied from 34.3 mg/kg (November) to 76.6 mg/kg (December), at NC ranged between 25.4 mg/kg (July) and 67.4 mg/kg (September) and at ChB from 52.7 mg/kg (March) to 61.9 mg/kg (July). The seasonal average concentration of Ni in sediment reduced progressively from monsoon onwards at CB and NC (Figure 4.2).

4.3.2.2 Suspended Particulate Matter (SPM)

The observed values of Ni in SPM at CB, NC and ChB were 108.7 mg/kg, 106.8 mg/kg and 116.7 mg/kg, respectively. At all the three sites, throughout the study period, on an average particulate Ni in SPM was found to be two fold higher than total Ni loading in sediment. The recorded values of Ni were more in monsoon (particularly in July), which decreased progressively with onset of pre-monsoonal months (Figure 4.2).

4.3.2.3 Seawater (Dissolved)

Average concentration of dissolved Ni content in CB, NC and ChB waters was 0.55 µg/l, 0.58 µg/l and 0.53 µg/l, respectively. Seasonal average of dissolved Ni exhibited more concentration

in monsoon and less in non-monsoon at CB and ChB waters whereas concentration showed opposite trend at NC (Figure 4.2).

4.3.2.4 Oyster Tissue

The average value of Ni accumulation in *C. madrasensis* tissue was found to be 0.88 mg/kg, 0.85 mg/kg and 3.11 mg/kg at CB, NC and ChB, respectively. At CB, tissue concentration varied from 0.12 mg/kg (September) to 4.51 mg/kg (March). At NC, it ranged between 0.19 mg/kg (August) and 1.93 mg/kg (April 2014). At ChB, concentration was much larger, minimum concentration was 1.66 mg/kg in November and maximum 5.61 mg/kg in March.

The average Ni concentration in the tissue of *C. gryphoides* was 0.82 mg/kg, 0.94 mg/kg and 1.31 mg/kg at CB, NC and ChB, respectively. Variation of Ni content at CB ranged from 0.23 mg/kg (June) to 1.6 mg/kg (April 2014). At NC highest value (3.18 mg/kg) was obtained in March and lowest (0.28 mg/kg) in August. While at ChB, Ni concentration varied between 1.15 mg/kg (November) and 1.46 mg/kg (July). Overall, Ni accumulation in oyster tissue was high in pre-monsoon (Figure 4.2).

4.3.3 Concentration of Pb

4.3.3.1 Surface Sediment

Average of Pb content (5.11 mg/kg) in CB sediment was higher (with no much seasonal variations) than at NC (1.23 mg/kg) and at ChB (2.77 mg/kg). On other hand, on seasonal scale sediment of NC and ChB showed higher Pb content in monsoon and lower in non-monsoon

(Figure 4.3). Unlike at CB, at NC and ChB its concentration varied widely from 0.85 mg/kg (June) to 1.87 mg/kg (September) and from 2.2 mg/kg (March) to 3.6 mg/kg (July), respectively.

4.3.3.2 Suspended Particulate Matter (SPM)

Average concentrations of particulate Pb at CB (3.49 mg/kg), NC (2.66 mg/kg) and ChB (2.08 mg/kg) did not show much variations. Overall, particulate Pb in surface seawaters was comparatively high at CB and ChB in monsoon and at NC in pre-monsoon 2. At NC, it was intriguing to find out that, concentrations of Pb in SPM were much higher than its total loading in surface sediment (Figure 4.3).

4.3.3.3 Seawater (Dissolved)

Dissolved Pb content at CB ranged from 0.03 µg/l (May, 2014) to 3.01 µg/l (July) (avg. 0.57 µg/l). At NC seawaters Pb values showed variation from 0.04 µg/l (May, 2014) to 2.05 µg/l (April, 2013) (avg. 0.41 µg/l). Whereas, Pb content in ChB seawater was low recorded between 0.04 µg/l (November) and 0.73 µg/l (July) (avg. 0.28 µg/l). On the whole, at all sites, dissolved Pb content was high in monsoon and low in non-monsoon (Figure 4.3).

4.3.3.4 Oyster Tissue

Comparatively similar bioaccumulation of Pb in *C. madrasensis* and *C. gryphoides* tissue was observed at all study sites. In tissue of *C. madrasensis,* Pb ranged from 0.10 mg/kg (June) – 0.81 mg/kg (December) at CB, 0.15 mg/kg (May 2013) – 1.10 mg/kg (May 2014) at NC and 0.24 mg/kg (March) – 0.84 mg/kg (November) at ChB. In the case of *C. gryphoides* tissue, it varied from 0.01 mg/kg (January) – 0.94 mg/kg (October) at CB, 0.03 mg/kg (January) – 1.15 mg/kg

(September) at NC and 0.29 mg/kg (November) – 0.31 mg/kg (July) at ChB. Seasonal variability in Pb accumulation in oyster tissue from all three sites showed its high level in non-monsoon and less in monsoon period (Figure 4.3).

4.3.4 Concentration of Cd

4.3.4.1 Surface Sediment

Average concentrations of Cd in surface sediment showed almost similar levels at CB (0.39 mg/kg), NC (0.30 mg/kg) and ChB (0.37 mg/kg) with highest concentration in monsoonal months. At CB and NC sediment, Cd levels varied from 0.20 mg/kg (March and May 2014) – 1.78 mg/kg (September) and 0.08 mg/kg (June) – 0.73 mg/kg (August), respectively. Similarly at ChB, values ranged from 0.31 mg/kg (November) to 0.45 mg/kg (July) (Figure 4.4).

4.3.4.2 Suspended Particulate Matter (SPM)

NC waters showed a higher level of particulate Cd (avg. 0.47 mg/kg) followed by CB (avg. 0.41 mg/kg) and ChB (avg. 0.28 mg/kg) waters. Seasonally, during monsoon maximum particulate Cd level was noticed at both NC (0.69 mg/kg; July) and at CB (0.57 mg/kg; August). While minimum values recorded in non-monsoonal period; 0.29 mg/kg (April 2013) and 0.32 mg/kg (May 2013 and February 2014) in non-monsoon period at NC and CB, respectively. In ChB waters, particulate Cd content varied from 0.23 mg/kg (November) to 0.35 mg/kg (March). Unlike ChB, values of particulate Cd was high in CB and NC waters than total Cd content in sediment (Figure 4.4).

4.3.4.3 Seawater (Dissolved)

High annual average of dissolved Cd found in ChB waters (0.24 µg/l) was higher than at CB (0.21 µg/l) and NC (0.18 µg/l). Overall, dissolved Cd in surface seawaters from all three sites were higher in non-monsoon and lower in monsoon period. At ChB site, values ranged from 0.14 µg/l (July) to 0.42 µg/l (November). Comparable ranges of dissolved Cd were recorded at CB (0.08 µg/l; August – 0.45 µg/l; October) and at NC, (0.08 µg/l; October – 0.47 µg/l; April, 2014) (Figure 4.4).

4.3.4.4 Oyster Tissue

The average value of Cd accumulation in *C. madrasensis* tissue was noted to be high (43.6 mg/kg) at ChB than other two sites CB (39.7 mg/kg), NC (18.14 mg/kg). Similar trend of results were obtained for *C. gryphoides*; average values at ChB (53.3 mg/kg) and CB (17.8 mg/kg), were higher that at NC (14.9 mg/kg). Overall, accumulation of Cd in oyster tissue at all three sites noticed to be high in monsoon and low in pre-monsoon (Figure 4.4).

4.3.5 Statistical Analysis

4.3.5.1 Correlation between the Metals Concentration in Oysters and the Metals Concentration in Sediment, SPM, Seawater (Dissolved) with Other Physico-chemical Variables

Pearson correlation test was used as a first approach to explore the possible relationships between the environmental parameters and metal concentration in sediment, particulate and dissolved metal in surface seawater, and in oyster tissue at three study sites. At CB, significant (p<0.05) positive correlation was recorded between sediment Ni and silt (r = 0.66), clay (r = 0.63) and TOC (r = 0.71) (Table 4.2 a). Moreover, sediment Cu and Cd showed significant

correlation coefficient with each other (r = 0.98, $p<0.001$) and with POC (r = 0.84 and 0.81, respectively $p<0.01$) in CB waters. Concentration of Cu and Cd in sediment showed significant ($p<0.05$) positive relationship with each other, with the Cd concentration in both the oyster species, also and with the Cu and Pb concentration in SPM (Table 4.2 b). At NC, accumulation of Pb in tissue of *C. madrasensis* was positively correlated with Chl *a* (r = 0.89, $p<0.05$), TSS (r = 0.94, $p<0.01$). Whereas, Pb content in *C. gryphoides* strongly associated ($p<0.05$) with pH (r = -0.86), Chl *a* (r = 0.88) and nutrients (NO_3+NO_2) (Table 4.3 a). Ni, Pb and Cd level in sediment also showed significant positive association with TOC (r = 0.92, r = 0.84, r = 0.82 respectively, $p<0.05$). Further, concentration of Pb and Cd in sediment was found positively ($p<0.05$) correlated with their concentration in SPM (Table 4.3 b). At ChB, Cd content in *C. madrasensis* had significant ($p<0.05$) positive relationship with DO (r = 1.00) and negative relationship with TOC and TC (r = -1.00) during study period. While, Ni and Pb accumulation in *C. gryphoides* negatively correlated ($p<0.05$) with silt (r = -1.00) and clay (r = -1.00), respectively (Table 4.4 a). In addition, Cd content in *C. madrasensis* positively correlated with particulate Cu (r = 1.00, $p<0.01$). While, Cu and Cd concentration was strongly correlated with each other (r = 1.00, $p<0.05$) in *C. gryphoides* (Table 4.4 b).

4.3.5.2 Analysis of Variance

Results of PERMANOVA showed a significant variation ($p<0.05$) in metals concentrations across all the seasons, sites, and substrates (sediment, seawater, SPM and tissue) as well as the interactions among these three main effects (Table 4.5). Since there was a significant interaction between these three factors, it has been further tested for two way PERMANOVA for sediment, SPM, water and three way PERMANOVA with two oyster species separately. A considerable

significant (p<0.05) sitewise variations were noticed in sediment metal concentrations (Table 4.6). Metal concentrations in SPM varied spatially and temporally and with their main effect interactions (Table 4.6). Dissolved metal concentration depicted only seasonal significant (p<0.05) variation (Table 4.6) whereas, significant species-wise and season-wise variation was obtained in metal accumulation in both the oyster species (Table 4.6).

4.3.5.3 Multivariate Analysis of Metals Concentration in Oyster Species, Sediment, SPM, Seawater (Dissolved) with other Physico-chemical Variables

RDA was used to relate the pattern of metal bioaccumulation in oyster species with measured environmental variables in various seasons. The length of the arrows (environmental variables) and their orientation indicates their relative importance and approximate correlations to the axes. Based on Forward selection and Monte Carlo permutation test pH, salinity and particulate Ni were identified as a statistically significant (p<0.05) variables that best explained the variation in metal accumulation in oyster species (marginal and conditional effect) (Table 4.7). Eigenvalues and cumulative percentage variance of data species and of species-environment relationship for surface seawater variables, particulate and dissolve metal variables as well as sediment variables, are given in (Table 4.8). Species scores and inter set correlation of environmental variables with axis given in (Table 4.9). Since the large data points on single plot are not clearly evident following three distinct RDA plots are drawn separately to make the data more understandable.

Water variables RDA plot (Figure 4.5) depicted pH and salinity correlate negatively, whereas DO and phosphate (moderately) correlate positively with the first axis. Axis 1 defined by positive score of Cu and Cd with *C. madrasensis*, and, Cu, Ni and Cd with *C. gryphoides*. While,

axis 2 depicts positive score of Cu on *C. madrasensis*. Sediment variables RDA plot (Figure 4.6) describes TOC and TC that are positively associated with axis 1 and sediment Ni was negatively associated with axis 2. Further, axis 1 distinct by negative score of Cd on *C. madrasensis*, Cu and Cd on *C. gryphoides*. Axis 2 characterized by positive score of Cu on *C. madrasensis*. Particulate and dissolve metal variables RDA plot (Figure 4.7) explains particulate Cu and Ni strongly and particulate Pb moderately correlated on first axis while particulate Pb and Cd positively associated with Axis 2. Axis 1 defined by positive score of Cd on *C. madrasensis*, and Cu, Ni and Cd on *C. gryphoides*. Axis 2 characterized by positive score of Cu on *C. madrasensis*.

4.4 Discussion

The natural environment is more complex than controlled systems, thus relationships between environmental variables and associated biota are difficult to pinpoint. Besides the natural processes, additional anthropogenic effluents coming from the industrial and mining activities contaminate the estuarine and coastal waters by enriching metal concentrations in sediments and water (Rath et al., 2009). Further, heavy metals are involved in an enormous range of chemical reactions which can also influence the biogeochemical cycle in the marine environment (Sen Gupta et al., 1978). In present study, it is noteworthy that the metal levels observed in all the measured components (surface sediment, SPM, surface seawater (dissolved) and oyster tissues) at ChB was similar in range when compared to CB and NC sites. This suggests that the reference site is also impacted by anthropogenic activities probably due to the nearby associated major fish landing jetty and sewage disposal from land inhabitants along the bank of the river Chapora.

4.4.1 Metals in Surface Sediment

Surface sediment at all the three sites showed enrichment of Cu (Figure 4.1) and Ni (Figure 4.2), exceeding the Effect Range Low (ERL) value (34 and 20 mg/kg, respectively) as per Sediment Quality Guideline (SQG) (Burton, 2002). Contrary, average values of Pb (Figure 4.3) and Cd (Figure 4.4) in sediment were much lower than the ERL limits (46.7 and 1.2 mg/kg, respectively) (Burton, 2002). Due to the absence of local and regional sediment quality guidelines for toxicity estimation of the measured heavy metal concentrations, ERL values were used for comparison. This comparison points out that the surface sediment of estuarine and coastal region of Goa is highly contaminated with Cu and Ni. The presence of ship and barge building industries, yards, workshops and other recreational anthropogenic activities and direct exposure to iron ores transportations at CB may be responsible for enrichment of metals in the surface sediment. Chipping, welding and antifouling painting works are being carried out at the ship-building unit throughout the year. Cu and Pb is being used in antifouling paints, whereas Ni is being used in welding galvanizing, and metal plating works (Shynu et al., 2012). Apart, occurrence of metals is also most likely arising from antifouling paint residues coming from the regular use of fishing trawlers, tourist boats and sewage coming from land based activities at NC and ChB. The results of present study is consistent with earlier studies conducted in the estuarine waters of Goa (Alagarsamy, 2006; Veerasingam et al., 2015; Chakraborty et al., 2015).

A significant ($p<0.05$) site wise variations in sediment metal concentrations can be attributed to different residence time of water column over the sediments, textural difference, sediment metabolism, and characteristics of the water at these sites. It has been reported that physicochemical properties of overlying water column (Coakley et al., 1993; Bruder et al.,

2002), freshwater discharge (Forstner and Whittmann, 1981), flow rates (Schoellhamer, 1995) and geomorphology of the area (Viles and Spencer, 1995) alter the metal distribution and speciation in estuarine sediment. The reason behind fluctuations of metal levels in CB and ChB sediment throughout the year could be due to the influence of geomorphology. The funnel shaped structure of the estuarine region (sampling site at near mouth) results the shifting of the surface sediment on regular basis under high velocity of wave, current and tidal flow. At NC, shallow and narrow basin structure results in less tidal impact and weak tidal current. Thus, the limited recipient capacity could be responsible for the lower metal concentrations at NC.

There are various ways through which heavy metals are fixed to the sediments such as direct adsorption by inorganic particles of clays, co-precipitation with hydroxides and iron oxides, adsorption or complexation with organic matter associated with inorganic particles, and other natural mechanisms related to the physico-chemical characteristics of sediments (Bruder et al., 2002). A significant positive correlation between sediment Ni and silt, clay and TOC in CB sediments was recorded (Table 4.2 a). These results indicate that accumulation of Ni in sediments are dependent on presence of finer particles (silt and clay) which hold more organic carbon in the sediments. The positive mutual association of studied metals and with TOC suggests that metal contaminants are contributed by same sources and also associated with fine sediment particles and organic matter. Wang et al. (2010) have found significant relation of Pb, Cu and Cd with organic matter. Surface sediment showed higher metals concentrations in all the seasons at CB and only in monsoon period at NC and ChB. This variability can be explained by the presence of the variations in sediment texture of the sites. CB contains comparatively more clayey sediment, which has a strong capacity to hold the metals whereas NC and ChB has more

of sandy sediment which has less affinity towards the metals. At NC, all the metals in sediment showed significant negative relation with salinity ($p<0.01$) and positive relationship with NO_3+NO_2, PO_4 and POC ($p<0.05$). This explains that the study area is mainly influenced by heavy rainfall events during SW monsoon (Figure 3.2 d). The heavy rainfall lead to increase in terrestrial runoff and thus freshwater inputs as well as metal transfer from the terrestrial to estuarine sediments. Sediment Cu and Cd showed significant correlation coefficient with each other ($p<0.0001$) and with POC ($p<0.01$) in CB waters which signifies that the sediment Cu and Cd level increases with rise in POC content mainly during monsoon. This is attributed to the Cu and Cd coming along with particulate organic carbon with monsoonal land runoff to aquatic system which ultimately settles to the bottom. Aguilar et al. (2012) found that sediment characteristics and presence of organic matter have a more influence on the transport and availability of heavy metals in rainy season.

4.4.2 Particulate Metals in Surface Seawater

Surface sediments often undergo resuspension into the water column due to both natural and anthropogenic processes like tides, currents, bioturbation, shipping, and dredging. SPM in the water column contains allochthonous sediment particles, organic material resuspended from the surface sediment and autochthonous biological particles (e.g., phytoplankton) to which various metals bind (Peakall and Burger, 2003). As suspended matter is well known for a metal scavenger, sediment resuspension significantly increases the risk of trace metal contaminant exposure to marine biota mainly to filter feeders (Lee et al., 2013).

Like sediment, particulate Cu and Ni also showed very high concentrations at all sites. The particulate Cu and Ni concentrations reported in present study was very good agreement with the values observed by Kessarkar et al. (2013) from the same estuarine region. Earlier published works also showed the high stability of Cu and Ni with SPM enrichment, where metal source coming from industrial effluent discharges (Hamad et al., 2012; Xu et al., 2015). Furthermore, particulate metals concentrations were recorded to be high in monsoon and low in pre-monsoon. This is supported by the statistical Pearson correlation coefficient results. Metal concentrations associated with SPM at all sites showed significant ($p<0.05$) negative correlation with water temperature, pH, salinity and positive correlation with nutrients and POC. This attributed to the input of large amount of organic and nutrient rich terrigenous land runoff that bring metal bound suspended particles from adjoining areas into the marine water bodies during rainy period. Also, this may be associated with estuarine turbidity maximum (ETM) resulting from resuspension phenomenon lead to high SPM content in estuarine waters of Goa (Rao et al., 2011). In addition, metals associated with surface sediment get mobilized during oxidizing condition (at low pH during monsoon) and resuspension event which further bind with suspended particle available in water column. Earlier studies from present study region have also reported high concentration of Fe and Mn content (Alagarsamy, 2006; Mesquita and Kaisary, 2007; Shynu et al., 2013). Therefore, there is a high possibility that Fe-Mn oxyhyroxides particles are the main factor which hold more metal content and increases the particulate metal concentrations in waters column (Chapman et al., 1998). In case of Cd, it is likely to remain in dissolve phase in overlying solution possibly due to the complexation with chlorides and other anions which can limit the sorption of Cd onto Fe oxides/hydroxides (Caetano et al., 2003; Xu et al., 2015).

4.4.3 Dissolved Metals in Surface Seawater

Total dissolved metal concentrations varied spatio-temporally due to variation in their transport processes, current pattern, mixing patterns (Jiann et al., 2013). In the present study, dissolved metals concentration was found to be very less than the metal concentrations recorded in other components (i.e. surface sediment and SPM) and showed significant ($p<0.01$) seasonal variations at all sites. Dissolved metals in seawater tends to form complexes with inorganic and organic ligands, which might be the reason for less concentration of dissolved metals in seawater (Balkis et al., 2010). Higher dissolved Cu, Ni and Pb concentrations occurred in monsoon (except at NC where high level of Cu and Ni recorded in pre-monsoon). During monsoon, low saline low pH waters increases the availability of divalent metals (bioavailable form) in seawaters. This is also proved by the statistical correlation results. Dissolve Ni and Pb (at CB), and dissolved Pb (at NC and ChB) showed significant negative relation with water temperature, pH, salinity and Chl *a* (in case of dissolve Ni) while positive correlation with nutrient loads, TSS and POC (at NC). This relationship explains the association of positively charged free ionic metals with negatively charged TSS/SPM (including clay, carbonate, oxide hydroxide of the Fe and Mn) and/or POC and its input indicates its terrigenous origin in monsoon (Dessai and Nayak, 2009; Shynu et al., 2015). However, in pre-monsoon rising pH increases the possible co-adsorption or co-precipitation of dissolve metals with particulate form of Fe-Mn or with other metal binding substrate like organic matter in water column (Tait and Dipper, 1998). But incase at NC, narrow geographic structure may contribute in high availability of dissolved metals due to less mixing and dilution of dissolved metal in pre-monsoon therefore dissolve metal content recorded to be comparatively high. Moreover, dissolve Cu found more compared to other dissolved metals content. High concentration of Cu in dissolve form may be due to either removal from suspended

matter by desorption in water column or its regeneration because of early diagenesis at the sediment and water interface (Bruland, 1983). Dissolve organic matter is known to bind and form stable complexes with dissolved trace metals such as Cu, Cd and Ni and sequestering them in water column (Vraspir and Butler, 2009).

4.4.4 Metals in Oyster Tissue

Metal concentrations in tissues of aquatic invertebrates living in the same habitat shows species specific response due to differences for metal uptake and accumulation (Rainbow, 2002 and references therein). In the present study, metal accumulation in oyster tissue showed significant species-wise and season-wise variation. Overall, *C. madrasensis* accumulated more metal contaminants than *C. gryphoides* though both species were exposed to the same water conditions (temperature, salinity, pH and type of food). This may be attributed to seasonal variations in oyster physiology and metabolism (Frazier, 1975; Guzman-Garcia et al., 2009; Barrera-Escorcia and Wong-Chang, 2010; Hariharan et al., 2014). These results reveal that species of same genus and from the same site can accumulate significantly different concentrations of the same element. Similar results were observed between two oyster species *C. corteziensis* and *C. palmula* from the same lagoon in Gulf of California accumulated different metal concentrations (Paez-Osuna and Osuna-Martinez, 2015).

Oysters are normally considered as a strong net accumulator for Cu (Rainbow et al., 1990) which is used in the synthesis of haemocyanin, a blood pigment (Yap et al., 2010). In present study, oyster tissues showed enrichment of Cu which is 12 times higher than the recommended limit 32 mg/kg set by FAO guideline (1983). Further, average seasonal values indicated maximal Cu accumulation in monsoon period in both the species at CB and ChB, and at NC in non-monsoon.

The Cu enriched sediment and SPM at the study sites in monsoon could be one of the main source for Cu enrichment in tissues of oyster. It is well known that the fine suspended particles of bottom sediment and ingestion of these resuspended particles from overlying water column forms important food source of the oyster. Since high concentrations of Cu (2100–4400 mg/kg dry wt.) cause oyster's mortality (Hung and Han, 1990), the excessive concentration of Cu noticed in current study indicates a higher risk to oysters along the Goa coast. These values corroborate with the Cu concentrations reported from other oyster species (Vasquez et al., 1993; De Mora et al., 2004; Fuad et al., 2013; Birch et al., 2014; Sarmadian et al., 2014; Bray et al., 2015). Moreover, consumption of oyster tissue as seafood with such a high concentration of Cu can lead to stunted growth, cirrhosis of the liver and jaundice in humans (Gorman, 1993).

Although the sediment and particulate matter from the ambience of oyster beds are highly polluted with Ni, the concentrations of Ni detected were well below the acceptable limit (70–80 mg/kg) (USFDA, 1993). This suggests that oyster has a good inequity against Ni. In addition, this explains that the Ni associated with sediment and SPM are probably not toxic to the studied species. Though the Ni concentration is far below the permissible limit, the measured levels of Ni were comparatively high in non-monsoon. This higher accumulation of metals might be attributed to the increase in surrounding surface seawater temperature during pre-monsoon season. The rise in surrounding temperature increases the metabolic activities, resulting in higher filtration rate, larger collection of suspended matter and higher uptake of heavy metals (Belivermis et al., 2016).

Likewise, the average concentration of Pb was below the recommended limit of 1.0 mg/kg (EU, 2001), but few samples exhibited values >1.0 mg/kg. This indicates that oyster from study sites are contaminated by Pb to some extent during non-monsoon period. This Pb accumulation may be due to the increase in traffic load of motor vehicles, boating activities, ships, combustion of fossil fuel, and organic waste discharge in recent times. Similarly, high concentration of Pb has been observed in sediment (Siraswar and Nayak, 2011; Veerasingam et al., 2015) and in Clam *Paphia malabarica* (Krishna Kumari et al., 2006) from the estuaries of Goa. Pb mainly affects the central nervous system, renal, haemapoietic, cardiovascular system of the human being (Flora et al., 2012). Some studies from elsewhere, have shown significant amounts of Pb accumulation in the soft tissues of oysters (Turkmen et al., 2005; Alfonso et al., 2013; Vazquez-Boucard et al., 2014; Bazzi, 2014).

Tissue of both the oyster species at all three sites showed elevated levels of Cd concentration with exceeding acceptable limit of 0.5 mg/kg set by FAO guideline (1983). Despite very low concentrations in ambience (sediment and particulate as well as dissolve form of Cd), high accumulation of Cd in oyster tissues at studied sites during monsoon were noticed. Chakraborty et al. (2016) found high bioaccumulation of Cd in *Crassostrea* sp. even at low Cd loading in bottom sediment from same study region. This enrichment of Cd in oyster tissues may be attributed to three different reasons. First, although heavy rainfall during monsoon period dilutes the metal concentrations in the surrounding waters, decrease in salinity (i.e. less chloride concentration) during monsoon increases the bioavailable form (free ion) of Cd to filter feeders (Engels and Fowler, 1979; Rainbow, 1995) and thus higher metal uptake by oysters. Second, Cd (II) mimics calcium ion Ca (II), via Ca^{2+} channels, (which is also abundant in seawater) due to its

similar geochemical properties, particularly ionic radius (cf. Ca 9.7 and Cd 9.8 nm) (Roesijadi and Unger, 1993; Williams and Frausto da Silva, 1996; Huanxin et al., 2000). Third, Cd has strong affinity towards the sulfhydryl groups of protein, the metallothionein (MT) and sequestered metal in detoxified form like Cu in intracellular granules (Rainbow, 2002; Apeti et al., 2005a) and less excretion, resulting in high concentration in tissue. Results of present study is further corroborated by previous studies where authors have found high concentrations of Cd in clam *P. malabarica* (1.4–8.4 mg/kg) along the Goa coast (Krishna Kumari et al., 2006) and in oyster *Saccostrea cucullata* (10–40 mg/kg) along Deltaic Sunderbans (Sarkar et al., 1994). Biomonitoring studies conducted on other oyster species elsewhere also observed the high concentration of Cd in their tissue (Presley et al., 1990; De Mora et al., 2004; Apeti et al., 2009; Heidari et al., 2013; Sarmadian et al., 2014). Cheng and Gobas (2007) have been reported that the Cd intakes of 0.43–0.71 μg/kg/day cause a toxic effect on consumers mainly to high risk groups, including women with low iron stores, people with renal impairment, smokers and children.

4.4.5 Relationship between the Metals Concentration in Oyster Species, Surface Sediment, SPM, Surface Seawater and other Physico-chemical Variables

The bioaccumulation of heavy metals in aquatic organisms depend on their chemical properties, bioavailability, and physico-chemical factors such as pH, salinity, DO, POC, nutrients, and SPM levels (Depledge and Rainbow, 1990). The negative association of metal accumulation in oyster with pH, salinity and positive correlation with DO and nutrients (Figure 4.5) demonstrates that hydrodynamics has a tendency to influence the oyster's metal uptake and its accumulation over a period of time. It is noted that pH as a major factor, and salinity as a second gradient contributing

to the metal enrichment in oyster. The low pH and salinity during monsoon season generally elevates the dissolve form of metals. The low pH and salinity observed during monsoon could be the reason for higher accumulation of Cu, Cd and to some extent Ni in tissue at CB and ChB. This observations is agreement with the results obtained by Yu et al. (2013). Moreover, since, west coast of India is characterized by upwelling event (Madhupratap et al., 2001), influence of upwelling may be another reason for Cd (than other metal) availability in oysters as suggested by Paez-Osuna and Osuna-Martinez (2015). However, samples collected in pre- and post-monsoon depicted more impact of high water temperature, pH, salinity and Chl *a* for Ni and Pb accumulation in *C. madrasensis*. This indicates that, as mentioned above, in pre-monsoon season due to high atmospheric temperature metabolic rate of oyster increases which accelerates the metal uptake in their body (Lannig et al., 2006).

Further, the RDA diagram on sediment variables representing TOC, TC, silt and clay showed negative influence mostly on monsoonal samples (Figure 4.6). However, positive impact on most of the non-monsoonal samples such as CB PreM1, PreM2, NC PreM1 and PostM were observed. This suggests that in monsoon, metals associated with organic matter (TOC and TC) and fine particles (silt and clay) in sediment release from sediment in overlying water column and become easily bioavailable to oyster (Shynu et al., 2013; Lee et al., 2013). While in non-monsoonal period, bound metals get settled on surface of sediment when river flow velocity relaxes (Dassenakis et al., 1995) and thus become less available to oysters.

RDA ordination diagram indicates that the particulate metals have more influence on the metal accumulation in oysters than metals present in dissolved form (Figure 4.7). This indicates that

the bioavailable form of Cu and Cd may be associated with suspended particles at studied sites forms a primary source of metals in the tissue of oysters. This may be attributed to the sediment texture which is made up of consolidated firm rocks with loose boulders and rocks on which sand (>79 %) are deposited. Due to high sand deposits, less metal binding ligands (e.g. silt and clay) remain available. This implies that organic matter containing more metal binding ligands is left in suspension phase in water column during natural events such as tides, waves (Eggleton and Thomas, 2004). Consequently, most metal toxicants could easily interchange and disperse through aquatic ecosystems which is finally taken up by the filter feeders such as oyster.

4.5 Conclusion

This study concludes that estuarine (and coastal) regions along the Goa coast were highly contaminated with Cu and Ni. The concentration of Cu and Cd and to some extent Pb in oyster tissue were found above the permissible limit (recommended by international authorities for safe consumption by humans) during study period. Therefore, the public has advised to think twice before consumption of oysters from the study region to reduce/avoid health hazard as the region has potential to accumulate metal contaminants. Present study also concludes that metals associated with the particulate matter in water column is the main source of metal accumulation in oyster. To reduce the metal contamination at studied sites, the identification of metal source(s) is very much essential. Addionally, continuous monitoring, people awareness and a stringent government policy is required to control the metal pollution in the Goan coastal waters.

Table 4.1 Quality control performance check with the metals concentration (mg/kg) in certified reference material MAG-1 and DORM-4 on dry weight basis.

Metals	MAG-1 (Sediment and SPM)			Dorm-4 (oyster)		
	Estimated	Certified	Recovery %	Estimated	Certified	Recovery %
Cu	30.04 ± 0.47	30 ± 3	100.13	14.42 ± 1.01	15.90 ± 0.90	90.69
Ni	52.90 ± 0.54	53 ± 8	99.81	1.20 ± 0.01	1.36 ± 0.22	88.24
Pb	22.99 ± 0.73	24 ± 3	95.79	0.32 ± 0.03	0.416 ± 0.05	76.92
Cd	0.70 ± 0.26	0.20 ± 0.03	80.00	0.33 ± 0.03	0.306 ± 0.02	107.84

MAG-1 = Marine sediment certified reference material for trace metal, DORM-4 = Fish protein certified reference material for trace metal. Abbreviation: SPM = suspended particulate matter. Values are presented as an average (n = 3).

Table 4.2 a Pearson correlation coefficient (r value) between metals concentration in surface sediment, SPM, surface seawater (dissolved), oyster tissue and physico-chemical parameters at Chicalim Bay (CB) during April 2013 – May 2014.

Parameters	Water temp.	pH	Salinity	DO	Chl *a*	NO_3+NO_2	PO_4	POC	TSS	Sand	Silt	Clay	TOC	TC
Cu sed.	-0.20	0.56	-0.09	0.03	0.42	0.01	-0.10	**0.84**[b]	0.06	0.50	-0.51	-0.42	-0.44	-0.52
Ni sed.	-0.14	0.13	0.39	-0.29	-0.15	-0.33	-0.30	0.00	-0.25	**-0.65**[a]	**0.66**[a]	**0.63**[a]	**0.71**[a]	0.56
Pb sed.	-0.04	-0.31	-0.44	0.21	-0.08	0.43	0.24	-0.37	0.17	0.26	-0.27	-0.20	-0.34	-0.33
Cd sed.	-0.26	0.48	-0.09	0.10	0.31	0.03	-0.10	**0.81**[b]	0.11	0.40	-0.41	-0.34	-0.33	-0.42
Cu SPM	-0.13	0.22	0.05	0.35	0.16	-0.04	-0.25	0.27	0.04	-0.04	0.03	0.17	0.05	-0.18
Ni SPM	-0.47	-0.26	-0.54	**0.90**[c]	-0.12	**0.60**[a]	0.20	-0.08	0.43	-0.13	0.11	0.21	0.11	0.04
Pb SPM	0.01	0.40	0.01	-0.02	0.47	0.00	0.10	**0.59**[a]	0.21	0.33	-0.33	-0.33	-0.22	-0.36
Cd SPM	-0.57	0.23	**-0.66**[a]	0.44	0.20	**0.68**[a]	0.23	0.48	0.37	0.31	-0.32	-0.25	-0.39	-0.40
Cu dis.	-0.08	0.06	0.00	0.29	-0.06	-0.04	0.08	0.14	0.22	-0.01	-0.01	0.08	-0.17	-0.26
Ni dis.	**-0.63**[a]	**-0.74**[b]	**-0.58**[a]	0.27	**-0.64**[a]	**0.62**[a]	0.36	-0.14	0.56	-0.15	0.16	0.07	0.22	0.38
Pb dis.	**-0.58**[a]	**-0.61**[a]	**-0.83**[b]	0.53	-0.20	**0.83**[b]	**0.77**[b]	0.08	**0.90**[c]	0.15	-0.14	-0.19	-0.14	0.02
Cd dis.	0.29	-0.07	0.19	-0.39	0.18	-0.19	0.30	0.00	0.24	0.11	-0.09	-0.16	-0.04	0.09
Cu Cm	-0.52	-0.40	-0.41	0.35	-0.24	0.37	-0.01	0.18	0.33	-0.18	0.19	0.23	0.18	0.24
Ni Cm	0.03	-0.18	0.08	0.20	-0.05	-0.15	-0.20	-0.33	-0.10	-0.40	0.37	0.57	0.21	0.17
Pb Cm	0.30	0.27	**0.67**[a]	-0.41	0.05	**-0.65**[a]	**-0.67**[a]	0.09	-0.52	-0.38	0.38	0.39	0.43	0.33
Cd Cm	-0.37	-0.22	-0.36	0.38	-0.07	0.29	-0.05	0.46	0.36	0.27	-0.27	-0.20	-0.20	-0.13
Cu Cg	-0.26	0.02	-0.44	**0.69**[a]	0.03	0.42	-0.04	0.28	0.21	0.32	-0.34	-0.24	-0.42	-0.47
Ni Cg	0.27	-0.22	0.26	0.15	-0.35	-0.33	-0.54	-0.51	-0.45	-0.28	0.26	0.40	0.03	-0.07
Pb Cg	0.55	-0.27	0.44	-0.39	-0.28	-0.40	-0.29	-0.56	-0.42	-0.03	0.04	-0.08	0.15	0.14
Cd Cg	-0.29	0.10	-0.20	0.27	0.11	0.14	-0.09	**0.66**[a]	0.26	0.36	-0.36	-0.30	-0.28	-0.26

Significance level at $p<0.05$ = a, $p<0.01$ = b, $p<0.001$ = c. Temp. = Temperature (°C), DO = dissolve oxygen (mg/l), Chl *a* = chlorophyll *a* (µg/l), NO_3 = nitrate (µmol/l), NO_2 = nitrite (µmol/l), PO_4 = phosphate (µmol/l), POC = particulate organic carbon (µg C/l), TSS = total suspended solids (mg/l), TOC = total organic carbon (%), TC = total carbon (%), sand, silt and clay (%), Cm = *Crassostrea madrasensis*, Cg = *Crassostrea gryphoides*, sed. = sediment, dis. = dissolved, SPM = suspended particulate matter. Metals concentration in sediment, SPM, oyster tissue expressed in mg/kg, and in seawater (dissolved) expressed in µg/l.

Table 4.2 b Pearson correlation coefficient (r value) between metals concentration in surface sediment, SPM, surface seawater (dissolved) and metals concentration in oyster tissues at CB (Chicalim Bay) during April 2013 – May 2014.

Parameters	Cu sed.	Ni sed.	Pb sed.	Cd sed.	Cu SPM	Ni SPM	Pb SPM	Cd SPM	Cu dis.	Ni dis.	Pb dis.	Cd dis.	Cu Cm	Ni Cm	Pb Cm	Cd Cm	Cu Cg	Ni Cg	Pb Cg	Cd Cg
Cu sed.	1.00																			
Ni sed.	0.08	1.00																		
Pb sed.	-0.34	-0.44	1.00																	
Cd sed.	**0.98**[c]	0.12	-0.41	1.00																
Cu SPM	**0.58**[a]	0.38	0.00	**0.62**[a]	1.00															
Ni SPM	-0.12	-0.18	0.33	-0.04	0.36	1.00														
Pb SPM	**0.66**[a]	0.26	-0.10	**0.67**[a]	**0.63**[a]	-0.15	1.00													
Cd SPM	0.24	-0.33	0.25	0.22	0.02	0.53	0.02	1.00												
Cu dis.	0.26	-0.30	-0.15	0.34	0.29	0.20	0.03	-0.07	1.00											
Ni dis.	-0.18	-0.03	0.20	-0.08	-0.06	0.38	-0.22	0.19	0.11	1.00										
Pb dis.	-0.18	-0.32	0.29	-0.12	-0.18	0.43	-0.13	0.31	0.18	**0.80**[b]	1.00									
Cd dis.	-0.05	0.00	-0.07	-0.08	-0.16	-0.49	0.20	-0.39	0.22	0.10	0.15	1.00								
Cu Cm	0.16	0.40	-0.15	0.21	0.24	0.30	-0.07	0.18	-0.20	0.54	0.41	-0.31	1.00							
Ni Cm	-0.22	0.29	0.10	-0.25	0.23	0.22	-0.40	-0.25	0.10	0.02	-0.03	-0.06	0.41	1.00						
Pb Cm	0.27	**0.79**[b]	**-0.59**[a]	0.28	0.35	-0.40	0.33	-0.35	-0.33	-0.35	**-0.65**[a]	-0.03	0.26	0.12	1.00					
Cd Cm	**0.64**[a]	0.06	-0.22	**0.69**[a]	0.48	0.22	0.25	0.17	0.24	0.45	0.33	-0.15	**0.72**[b]	0.10	0.14	1.00				
Cu Cg	0.28	-0.53	0.14	0.33	0.19	**0.63**[a]	-0.04	**0.64**[a]	0.27	0.06	0.20	**-0.68**[a]	0.15	-0.20	-0.35	0.39	1.00			
Ni Cg	-0.24	-0.11	0.19	-0.23	0.11	0.17	-0.46	-0.28	0.21	-0.17	-0.25	-0.51	0.08	0.56	-0.01	0.02	0.30	1.00		
Pb Cg	-0.29	0.01	0.31	-0.29	-0.01	-0.32	0.18	-0.56	-0.28	-0.16	-0.32	0.05	-0.40	-0.28	0.14	-0.32	-0.25	0.21	1.00	
Cd Cg	**0.85**[c]	0.04	-0.40	**0.90**[c]	0.54	0.09	0.46	0.18	0.36	0.21	0.13	-0.09	0.47	-0.11	0.22	**0.92**[c]	0.41	-0.11	-0.33	1.00

Significance level at $p<0.05$ = a, $p<0.01$ = b, $p<0.001$ = c. Abbreviations: Cm = *Crassostrea madrasensis*, Cg = *Crassostrea gryphoides*, sed. = sediment, dis. = dissolved, SPM = suspended particulate matter. Metals concentration in sediment, SPM, oyster tissue expressed in mg/kg, and in seawater (dissolved) expressed in µg/l.

Table 4.3 a Pearson correlation coefficient (r value) metals concentration in surface sediment, SPM, surface seawater (dissolved), oyster tissue and physico-chemical parameters at NC (Nerul Creek) during April 2013 – May 2014.

Parameters	water temp.	pH	Salinity	DO	Chl *a*	NO_3+NO_2	PO_4	POC	TSS	Sand	Silt	Clay	TOC	TC
Cu sed.	**-0.84**[a]	-0.37	-0.67	0.38	0.14	0.53	0.67	0.72	-0.25	-0.22	0.25	0.08	0.73	0.66
Ni sed.	**-0.88**[a]	-0.26	-0.80	0.55	0.13	0.60	0.75	**0.85**[a]	-0.28	-0.44	0.46	0.32	**0.92**[a]	**0.83**[a]
Pb sed.	-0.77	-0.57	**-0.95**[b]	0.68	0.25	0.79	**0.88**[a]	**0.89**[a]	-0.16	-0.31	0.31	0.33	**0.84**[a]	0.67
Cd sed.	-0.67	-0.50	**-0.97**[b]	0.59	0.30	**0.84**[a]	**0.92**[a]	**0.95**[b]	-0.08	-0.38	0.39	0.38	**0.82**[a]	0.63
Cu SPM	-0.46	-0.16	-0.12	0.73	-0.61	-0.02	0.10	0.01	-0.73	-0.32	0.28	0.49	0.30	0.34
Ni SPM	-0.73	-0.62	-0.77	0.80	0.00	0.61	0.70	0.64	-0.36	-0.22	0.20	0.35	0.69	0.55
Pb SPM	-0.69	-0.23	**-0.83**[a]	0.38	0.27	0.69	0.81	**0.92**[b]	-0.09	-0.47	0.50	0.32	**0.82**[a]	0.70
Cd SPM	-0.39	-0.64	**-0.97**[b]	0.34	0.56	**0.94**[b]	**0.94**[b]	**0.94**[b]	0.25	-0.17	0.17	0.18	0.58	0.34
Cu dis.	0.15	-0.08	0.14	0.37	-0.05	-0.22	-0.29	-0.37	-0.01	0.24	-0.27	0.03	-0.05	-0.08
Ni dis.	0.24	-0.42	0.09	-0.51	0.74	0.00	-0.13	-0.21	0.74	**0.94**[b]	**-0.92**[a]	**-0.96**[b]	-0.32	-0.41
Pb dis.	-0.55	-0.52	**-0.97**[b]	0.41	0.36	**0.91**[a]	**0.97**[b]	**1.00**[c]	0.02	-0.38	0.39	0.34	0.70	0.50
Cd dis.	**0.82**[a]	-0.18	0.20	**-0.81**[a]	0.69	0.07	-0.15	-0.21	**0.93**[b]	0.65	-0.64	-0.66	-0.70	-0.80
Cu Cm	0.35	0.01	0.23	-0.12	-0.45	-0.07	-0.08	-0.19	-0.24	-0.19	0.15	0.26	-0.47	-0.42
Ni Cm	0.40	0.17	0.46	0.10	-0.64	-0.38	-0.41	-0.52	-0.37	-0.14	0.09	0.34	-0.49	-0.39
Pb Cm	0.42	-0.36	-0.02	-0.72	**0.89**[a]	0.20	0.03	0.00	**0.94**[b]	0.78	-0.76	**-0.87**[a]	-0.38	-0.51
Cd Cm	0.39	0.11	0.35	0.17	-0.59	-0.27	-0.30	-0.43	-0.36	-0.18	0.12	0.40	-0.43	-0.37
Cu Cg	-0.15	-0.09	-0.10	0.38	-0.59	0.11	0.19	0.10	-0.58	-0.46	0.42	0.56	0.03	0.06
Ni Cg	0.34	-0.02	0.33	-0.03	-0.50	-0.20	-0.22	-0.36	-0.29	-0.05	0.01	0.19	-0.50	-0.44
Pb Cg	-0.18	**-0.86**[a]	-0.73	-0.15	**0.88**[a]	**0.82**[a]	0.75	0.67	0.64	0.46	-0.44	-0.50	0.18	-0.06
Cd Cg	0.02	-0.09	0.12	0.57	-0.33	-0.22	-0.24	-0.34	-0.31	0.05	-0.09	0.26	0.00	0.01

Significance level at $p<0.05$ = a, $p<0.01$ = b, $p<0.001$ = c. Temp. = Temperature (°C), DO = dissolve oxygen (mg/l), Chl *a* = chlorophyll *a* (µg/l), NO_3 = nitrate (µmol/l), NO_2 = nitrite (µmol/l), PO_4 = phosphate (µmol/l), POC = particulate organic carbon (µg C/l), TSS = total suspended solids (mg/l), TOC = total organic carbon (%), TC = total carbon (%), sand, silt and clay (%), Cm = *Crassostrea madrasensis*, Cg = *Crassostrea gryphoides*, sed. = sediment, dis. = dissolved, SPM = suspended particulate matter. Metals concentration in sediment, SPM, oyster tissue expressed in mg/kg, and in seawater (dissolved) expressed in µg/l.

Table 4.3 b Pearson correlation coefficient (r value) between metals concentration in surface sediment, SPM, surface seawater (dissolved) and metals concentration in oyster tissues at NC (Nerul Creek) during April 2013 – May 2014.

Parameters	Cu sed.	Ni sed.	Pb sed.	Cd sed.	Cu SPM	Ni SPM	Pb SPM	Cd SPM	Cu dis.	Ni dis.	Pb dis.	Cd dis.	Cu Cm	Ni Cm	Pb Cm	Cd Cm	Cu Cg	Ni Cg	Pb Cg	Cd Cg
Cu sed.	1.00																			
Ni sed.	**0.91**[a]	1.00																		
Pb sed.	0.76	**0.89**[a]	1.00																	
Cd sed.	0.69	**0.87**[a]	**0.97**[b]	1.00																
Cu SPM	0.26	0.16	0.31	0.16	1.00															
Ni SPM	0.64	0.67	**0.88**[a]	0.77	0.70	1.00														
Pb SPM	0.78	**0.94**[b]	**0.83**[a]	**0.89**[a]	-0.09	0.50	1.00													
Cd SPM	0.51	0.70	**0.87**[a]	**0.93**[b]	-0.07	0.62	0.80	1.00												
Cu dis.	-0.55	-0.38	-0.05	-0.10	0.21	0.14	-0.43	-0.07	1.00											
Ni dis.	-0.06	-0.21	-0.17	-0.23	-0.49	-0.22	-0.23	-0.04	0.18	1.00										
Pb dis.	0.69	**0.84**[a]	**0.91**[a]	**0.96**[b]	0.06	0.67	**0.91**[a]	**0.94**[b]	-0.31	-0.25	1.00									
Cd dis.	-0.56	-0.56	-0.46	-0.35	-0.78	-0.62	-0.33	0.00	0.09	0.62	-0.24	1.00								
Cu Cm	-0.18	-0.38	-0.30	-0.33	0.48	-0.02	-0.40	-0.32	-0.28	-0.49	-0.18	-0.13	1.00							
Ni Cm	-0.53	-0.62	-0.44	-0.49	0.59	-0.04	-0.70	-0.51	0.28	-0.46	-0.48	-0.16	**0.82**[a]	1.00						
Pb Cm	-0.11	-0.18	-0.18	-0.13	-0.80	-0.41	-0.04	0.15	-0.06	**0.88**[a]	-0.06	**0.84**[a]	-0.46	-0.58	1.00					
Cd Cm	-0.52	-0.58	-0.34	-0.38	0.60	0.04	-0.63	-0.39	0.34	-0.50	-0.37	-0.15	0.80	**0.99**[c]	-0.59	1.00				
Cu Cg	0.18	0.04	0.14	0.08	0.81	0.45	-0.07	-0.06	-0.20	-0.70	0.14	-0.58	**0.85**[a]	0.72	-0.76	0.73	1.00			
Ni Cg	-0.26	-0.49	-0.35	-0.42	0.61	0.04	-0.58	-0.43	-0.04	-0.39	-0.34	-0.17	**0.96**[b]	**0.92**[a]	-0.48	**0.89**[a]	**0.84**[a]	1.00		
Pb Cg	0.54	0.46	0.58	0.57	-0.25	0.39	0.51	0.72	-0.24	0.54	0.63	0.31	-0.30	-0.61	0.62	-0.56	-0.25	-0.36	1.00	
Cd Cg	-0.42	-0.32	0.02	-0.08	0.58	0.35	-0.46	-0.13	**0.91**[a]	-0.10	-0.27	-0.21	0.06	0.56	-0.41	0.61	0.22	0.30	-0.36	1.00

Significance level at $p<0.05$ = a, $p<0.01$ = b, $p<0.001$ = c. Abbreviations: Cm = *Crassostrea madrasensis*, Cg = *Crassostrea gryphoides*, sed. = sediment, dis. = dissolved, SPM = suspended particulate matter. Metals concentration in sediment, SPM, oyster tissue expressed in mg/kg, and in seawater (dissolved) expressed in µg/l.

Table 4.4 a Pearson correlation coefficient (r value) metals concentration in surface sediment, SPM, seawater (dissolved), oyster tissue and physico-chemical parameters at ChB (Chapora Bay) during April 2013 – May 2014.

Parameters	water temp.	pH	Salinity	DO	Chl *a*	NO_3+NO_2	PO_4	POC	TSS	Sand	Silt	Clay	TOC	TC
Cu sed.	-0.40	-0.60	-0.64	0.22	-0.92	0.65	0.67	0.01	0.59	-0.12	0.10	0.32	-0.30	-0.20
Ni sed.	-0.94	-0.83	-0.81	0.99	-0.45	0.80	0.78	**1.00**[a]	0.84	0.98	-0.99	-0.93	-0.97	-0.99
Pb sed.	**-1.00**[a]	-0.99	-0.98	0.97	-0.77	0.98	0.97	0.89	0.99	0.83	-0.84	-0.69	-0.98	-0.96
Cd sed.	-0.80	-0.92	-0.94	0.67	-0.99	0.94	0.95	0.50	0.91	0.39	-0.41	-0.19	-0.73	-0.66
Cu SPM	-0.99	-0.92	-0.91	**1.00**[a]	-0.61	0.90	0.89	0.97	0.93	0.93	-0.94	-0.84	**-1.00**[a]	**-1.00**[a]
Ni SPM	-0.98	**-1.00**[a]	**-1.00**[a]	0.92	-0.85	1.00	0.99	0.82	**1.00**[a]	0.74	-0.75	-0.58	-0.95	-0.91
Pb SPM	**-1.00**[a]	-0.98	-0.97	0.98	-0.75	0.97	0.96	0.91	0.98	0.85	-0.86	-0.72	-0.99	-0.97
Cd SPM	0.54	0.33	0.28	-0.69	-0.18	-0.28	-0.24	-0.83	-0.34	-0.89	0.88	0.97	0.63	0.70
Cu dis.	-0.09	0.14	0.19	0.28	0.62	-0.20	-0.23	0.47	-0.13	0.58	-0.57	-0.74	-0.20	-0.30
Ni dis.	-0.49	-0.68	-0.71	0.31	-0.95	0.72	0.74	0.11	0.67	-0.02	0.00	0.23	-0.39	-0.29
Pb dis.	-0.95	**-1.00**[a]	**-1.00**[a]	0.88	-0.90	**1.00**[a]	**1.00**[b]	0.76	1.00	0.67	-0.68	-0.50	-0.91	-0.87
Cd dis.	0.27	0.49	0.53	-0.08	0.86	-0.53	-0.56	0.13	-0.48	0.25	-0.23	-0.45	0.16	0.06
Cu Cm	-0.28	-0.50	-0.54	0.10	-0.86	0.55	0.57	-0.12	0.49	-0.24	0.22	0.44	-0.18	-0.08
Ni Cm	0.65	0.46	0.42	-0.79	-0.04	-0.41	-0.38	-0.90	-0.47	-0.95	0.94	0.99	0.73	0.80
Pb Cm	0.07	0.30	0.34	0.12	0.73	-0.35	-0.38	0.33	-0.29	0.44	-0.43	-0.62	-0.04	-0.14
Cd Cm	-0.99	-0.93	-0.91	**1.00**[a]	-0.62	0.91	0.89	0.97	0.93	0.93	-0.94	-0.83	**-1.00**[a]	**-1.00**[a]
Cu Cg	**-1.00**[a]	-0.98	-0.97	0.97	-0.76	0.97	0.96	0.90	0.98	0.84	-0.85	-0.71	-0.99	-0.97
Ni Cg	-0.85	-0.70	-0.66	0.93	-0.25	0.66	0.63	0.99	0.71	**1.00**[a]	**-1.00**[a]	-0.98	-0.90	-0.94
Pb Cg	-0.76	-0.59	-0.55	0.87	-0.11	0.54	0.51	0.95	0.60	0.98	-0.98	**-1.00**[a]	-0.83	-0.88
Cd Cg	-1.00	-0.99	-0.98	0.96	-0.79	0.98	0.97	0.88	0.99	0.82	-0.83	-0.68	-0.98	-0.96

Significance level at $p<0.05$ = a, $p<0.01$ = b, $p<0.001$ = c. Temp. = Temperature (°C), DO = dissolve oxygen (mg/l), Chl *a* = chlorophyll *a* (µg/l), NO_3 = nitrate (µmol/l), NO_2 = nitrite (µmol/l), PO_4 = phosphate (µmol/l), POC = particulate organic carbon (µg C/l), TSS = total suspended solids (mg/l), TOC = total organic carbon (%), TC = total carbon (%), sand, silt and clay (%), Cm = *Crassostrea madrasensis*, Cg = *Crassostrea gryphoides*, sed. = sediment, dis. = dissolved, SPM = suspended particulate matter. Metals concentration in sediment, SPM, oyster tissue expressed in mg/kg, and in seawater (dissolved) expressed in µg/l.

Table 4.4 b Pearson correlation coefficient (r value) between metals concentration in surface sediment, SPM, surface seawater (dissolved) and metals concentration in oyster tissues at ChB (Chapora Bay) during April 2013 – May 2014.

Parameters	Cu sed.	Ni sed.	Pb sed.	Cd sed.	Cu SPM	Ni SPM	Pb SPM	Cd SPM	Cu dis.	Ni dis.	Pb dis.	Cd dis.	Cu Cm	Ni Cm	Pb Cm	Cd Cm	Cu Cg	Ni Cg	Pb Cg	Cd Cg
Cu sed.	1.00																			
Ni sed.	0.06	1.00																		
Pb sed.	0.46	0.91	1.00																	
Cd sed.	0.87	0.55	0.84	1.00																
Cu SPM	0.25	0.98	0.97	0.70	1.00															
Ni SPM	0.58	0.85	0.99	0.91	0.93	1.00														
Pb SPM	0.43	0.93	**1.00**[a]	0.82	0.98	0.98	1.00													
Cd SPM	0.56	-0.80	-0.48	0.07	-0.66	-0.35	-0.51	1.00												
Cu dis.	-0.88	0.43	0.02	-0.52	0.25	-0.12	0.06	-0.89	1.00											
Ni dis.	1.00	0.16	0.55	0.91	0.34	0.66	0.51	0.47	-0.83	1.00										
Pb dis.	0.66	0.79	0.97	0.95	0.89	0.99	0.96	-0.26	-0.22	0.73	1.00									
Cd dis.	-0.99	0.08	-0.34	-0.79	-0.12	-0.46	-0.30	-0.67	0.93	-0.97	-0.55	1.00								
Cu Cm	0.99	-0.07	0.35	0.80	0.13	0.47	0.31	0.66	-0.93	0.98	0.56	**-1.00**[b]	1.00							
Ni Cm	0.43	-0.88	-0.60	-0.07	-0.76	-0.48	-0.63	0.99	-0.81	0.34	-0.39	-0.55	0.54	1.00						
Pb Cm	-0.94	0.28	-0.14	-0.65	0.09	-0.27	-0.10	-0.80	0.99	-0.91	-0.37	0.98	-0.98	-0.71	1.00					
Cd Cm	0.26	0.98	0.98	0.70	**1.00**[b]	0.94	0.98	-0.66	0.24	0.35	0.90	-0.13	0.14	-0.76	0.08	1.00				
Cu Cg	0.44	0.92	**1.00**[a]	0.83	0.98	0.99	**1.00**[a]	-0.50	0.04	0.53	0.97	-0.32	0.33	-0.62	-0.12	0.98	1.00			
Ni Cg	-0.15	0.98	0.81	0.36	0.92	0.72	0.83	-0.91	0.61	-0.05	0.64	0.29	-0.27	-0.96	0.47	0.92	0.82	1.00		
Pb Cg	-0.29	0.94	0.71	0.22	0.85	0.61	0.74	-0.96	0.72	-0.20	0.52	0.42	-0.41	-0.99	0.60	0.85	0.73	0.99	1.00	
Cd Cg	0.48	0.90	**1.00**[a]	0.85	0.97	0.99	**1.00**[a]	-0.46	0.00	0.56	0.98	-0.35	0.37	-0.59	-0.16	0.97	**1.00**[a]	0.80	0.70	1.00

Significance level at $p<0.05$ = a, $p<0.01$ = b, $p<0.001$ = c. Abbreviations: Cm = *Crassostrea madrasensis*, Cg = *Crassostrea gryphoides*, sed. = sediment, dis. = dissolved, SPM = suspended particulate matter. Metals concentration in sediment, SPM, oyster tissue expressed in mg/kg, and in seawater (dissolved) expressed in µg/l.

Table 4.5 Results of PERMANOVA test for metals concentration in relation to seasons, sites, substrates and their interaction.

Factors	Df	SS	MS	Pseudo-F	*p* (MC)	Unique perm
season	3	3514.7	1171.6	3.745	*0.001*	999
site	2	4527.8	2263.9	7.2366	*0.001*	997
substrate	3	3.05E+05	1.02E+05	324.65	*0.001*	999
season × site	5	2210.7	442.15	1.4133	0.129	998
season × substrate	9	8056	895.11	2.8613	*0.001*	998
site × substrate	6	8387.3	1397.9	4.4684	*0.001*	998
season × site × substrate	15	5912.6	394.17	1.26	0.113	996
Residual	139	43484	312.84			
Total	182	4.45E+05				

Italic value indicates the significance level at $p<0.05$. Abbreviations: Df = degrees of freedom, SS = sum of squares, MS = mean sum of squares, Pseudo-F = F value by permutation, *p* values are based on 999 permutations based on Monte Carlo (MC). Seasons: Pre-monsoon 1, monsoon, post-monsoon, pre-monsoon 2; Sites: Chicalim Bay, Nerul Creek, Chapora Bay; Substrates: surface sediment, suspended particulate matter, surface seawater (dissolved), oyster tissue.

Table 4.6 Results of PERMANOVA test for metals concentration in relation to seasons, sites, and their interaction.

Substrate	Factors	Df	SS	MS	Pseudo-F	*p* (MC)	Unique perm
Sediment	season	3	1441.5	480.5	1.9252	0.069	999
	site	2	9904.5	4952.3	19.842	*0.001*	999
	season × site	5	1977.1	395.41	1.5843	0.116	999
	Residual	28	6988.4	249.58			
	Total	38	2.08E+04				
SPM	season	3	1456.7	485.57	19.533	*0.001*	999
	site	2	1644.1	822.06	33.07	*0.001*	999
	season × site	5	635.3	127.06	5.1114	*0.002*	998
	Residual	28	696.04	24.858			
	Total	38	4.60E+03				
Seawater	season	3	3469.9	1156.6	3.474	*0.002*	997
	site	2	302.48	151.24	0.45425	0.779	998
	season × site	5	2275.6	455.12	1.367	0.204	998
	Residual	28	9322.4	332.94			
	Total	38	1.55E+04				
Oyster	species	1	2918.4	2918.4	7.8203	*0.002*	995
	season	3	8121.1	2707	7.2538	*0.001*	999
	site	2	1267.1	633.56	1.6977	0.134	998
	species × season	3	3256.8	1085.6	2.909	*0.007*	998
	species × site	2	1314.9	657.45	1.7617	0.119	999
	season × site	5	3653.5	730.69	1.958	*0.050*	998
	species × season × site	3	2800.3	933.43	2.5012	*0.027*	998
	Residual	46	1.72E+04	373.19			
	Total	65	37302				

Italic value indicates the significance level at $p<0.05$. Abbreviation: Df = degrees of freedom, SS = sum of squares, MS = mean sum of squares, Pseudo-F = F value by permutation, p values are based on 999 permutations based on Monte Carlo (MC), SPM = suspended particulate matter. Seasons: Pre-monsoon 1, monsoon, post-monsoon, pre-monsoon 2; Sites: Chicalim Bay, Nerul Creek, Chapora Bay; oyster species: *Crassostrea madrasensis*, *Crassostrea gryphoides*.

Table 4.7 Result of redundancy analysis correlating the metals concentration in oyster tissue with physico-chemical parameters including metals concentration in sediment, SPM, surface seawater (dissolved) at study sites.

Marginal Effects		Conditional Effects		
Parameters	Lambda (1)	Parameters	Lambda (I)	*p* value
pH	0.41	pH	0.41	**0.011**
Salinity	0.32	DO	0.19	**0.047**
DO	0.26	Salinity	0.05	0.360
Water temp.	0.16	TSS	0.09	0.192
PO_4	0.14	Chl *a*	0.06	0.295
TSS	0.06	Water temp.	0.05	0.334
POC	0.03	PO_4	0.02	0.618
Chl *a*	0.03	POC	0.04	0.475
NO_3+NO_2	0.01	NO_3+NO_2	0.00	0.998
TOC	0.14	TOC	0.14	0.250
TC	0.14	Clay	0.08	0.383
Cd sed.	0.11	Cd sed	0.07	0.405
Pb sed.	0.09	Cu sed	0.09	0.414
Ni sed.	0.07	Pb sed	0.08	0.481
Cu sed.	0.05	Ni sed	0.12	0.328
Silt	0.04	Sand	0.03	0.745
Sand	0.04	TC	0.07	0.599
Clay	0.02	Silt	0.01	0.957
Ni SPM	0.50	Ni SPM	0.50	**0.002**
Cu SPM	0.44	Pb SPM	0.12	0.114
Pb SPM	0.18	Cd SPM	0.09	0.143
Cd dis	0.09	Cu SPM	0.05	0.302
Cd SPM	0.04	Cd dis	0.03	0.532
Pb dis.	0.02	Cu dis	0.05	0.333
Ni dis.	0.01	Pb dis	0.02	0.509
Cu dis.	0.00	Ni dis	0.02	0.678

Significance level at $p<0.05$. Temp. = Temperature (°C), DO = dissolve oxygen (mg/l), Chl *a* = chlorophyll *a* (µg/l), NO_3-N = nitrate (µmol/l), NO_2-N = nitrite (µmol/l), PO_4-P = phosphate (µmol/l), POC = particulate organic carbon (µg C/l), TSS = total suspended solids (mg/l), TOC = total organic carbon (%), TC = total carbon (%), sand, silt and clay (%), sed. = sediment, dis. = dissolved, SPM = suspended particulate matter. Metals concentration in surface sediment and SPM expressed in mg/kg, whereas in seawater (dissolved) expressed in µg/l. Lambda (I) is the eigenvalue explained by the environmental variable.

Table 4.8 Results of redundancy analysis related to eigenvalues for axes, species-environment correlations, cumulative percentage variance of species data and species-environmental relation.

	Axis 1	Axis 2	Axis 3	Axis 4
Surface seawater variables				
Eigenvalues :	0.798	0.109	0.005	0.001
Species-environment correlations :	0.978	0.830	0.898	0.733
Cumulative percentage variance				
of species data :	79.800	90.600	91.100	91.200
of species-environment relation:	87.400	99.300	99.900	100.000
Sediment variables				
Eigenvalues :	0.351	0.126	0.005	0.001
Species-environment correlations :	0.649	0.892	0.932	0.574
Cumulative percentage variance				
of species data :	35.100	47.700	48.300	48.400
of species-environment relation:	72.700	98.700	99.900	100.000
Particulate and dissolve metal variables				
Eigenvalues :	0.756	0.121	0.006	0.002
Species-environment correlations :	0.954	0.866	0.995	0.973
Cumulative percentage variance				
of species data :	75.600	87.700	88.300	88.500
of species-environment relation:	85.500	99.200	99.800	100.000

Table 4.9 Results of redundancy analysis related to species scores and Inter set correlations of environmental variables with axes.

Species scores (adjusted for species variance)				Inter set correlations of environmental variables with axes		
		Axis 1	Axis 2		Axis 1	Axis 2
Water variables						
	Cu Cm	**0.486**	**0.754**	Water temp.	-0.395	0.395
	Ni Cm	-0.069	-0.113	pH	**-0.686**	0.343
	Pb Cm	-0.014	-0.079	Salinity	**-0.603**	0.274
	Cd Cm	**0.639**	-0.199	DO	**0.562**	-0.074
	Cu Cg	**0.964**	-0.079	Chl *a*	-0.176	0.160
	Ni Cg	**0.717**	0.139	NO_3+NO_2	0.066	0.116
	Pb Cg	0.165	0.174	PO4	0.405	-0.046
	Cd Cg	**0.843**	-0.403	POC	0.175	0.224
				TSS	0.247	0.259
Sediment variables						
	Cu Cm	-0.327	**0.813**	Sand	-0.199	-0.185
	Ni Cm	-0.220	-0.042	Silt	0.209	0.184
	Pb Cm	-0.003	0.093	Clay	0.131	0.189
	Cd Cm	**-0.527**	0.007	TOC	**0.409**	-0.025
	Cu Cg	**-0.638**	-0.088	TC	**0.408**	0.057
	Ni Cg	-0.299	0.287	Cu sed.	-0.203	0.281
	Pb Cg	-0.237	-0.011	Ni sed.	-0.079	**-0.650**
	Cd Cg	**-0.621**	-0.403	Pb sed.	-0.323	0.007
				Cd sed.	-0.365	0.016
Suspended particulate matter and dissolve metal variables						
	Cu Cm	0.211	**0.816**	Cu SPM	**0.714**	-0.284
	Ni Cm	-0.140	0.065	Ni SPM	**0.771**	-0.140
	Pb Cm	-0.055	-0.049	Pb SPM	0.418	**0.445**
	Cd Cm	**0.619**	-0.056	Cd SPM	-0.088	**0.462**
	Cu Cg	**0.959**	-0.037	Cu dis	-0.022	0.100
	Ni Cg	**0.548**	0.158	Ni dis	-0.107	-0.063
	Pb Cg	0.160	0.215	Pb dis	0.138	-0.049
	Cd Cg	**0.837**	-0.269	Cd dis	-0.321	-0.207

Abbreviation: Temp. = Temperature (°C), DO = dissolve oxygen (mg/l), Chl *a* = chlorophyll *a* (µg/l), NO_3-N = nitrate (µmol/l), NO_2-N = nitrite (µmol/l), PO_4-P = phosphate (µmol/l), POC = particulate organic carbon (µg C/l), TSS = total suspended solids (mg/l), TOC = total organic carbon (%), TC = total carbon (%), sand, silt and clay (%), sed. = sediment, dis. = dissolved, SPM = suspended particulate matter, Cm = *Crassostrea madrasensis*, Cg = *Crassostrea gryphoides*. Metals concentration in SPM, sediment and oyster tissue expressed in mg/kg, whereas in seawater (dissolved) expressed in µg/l.

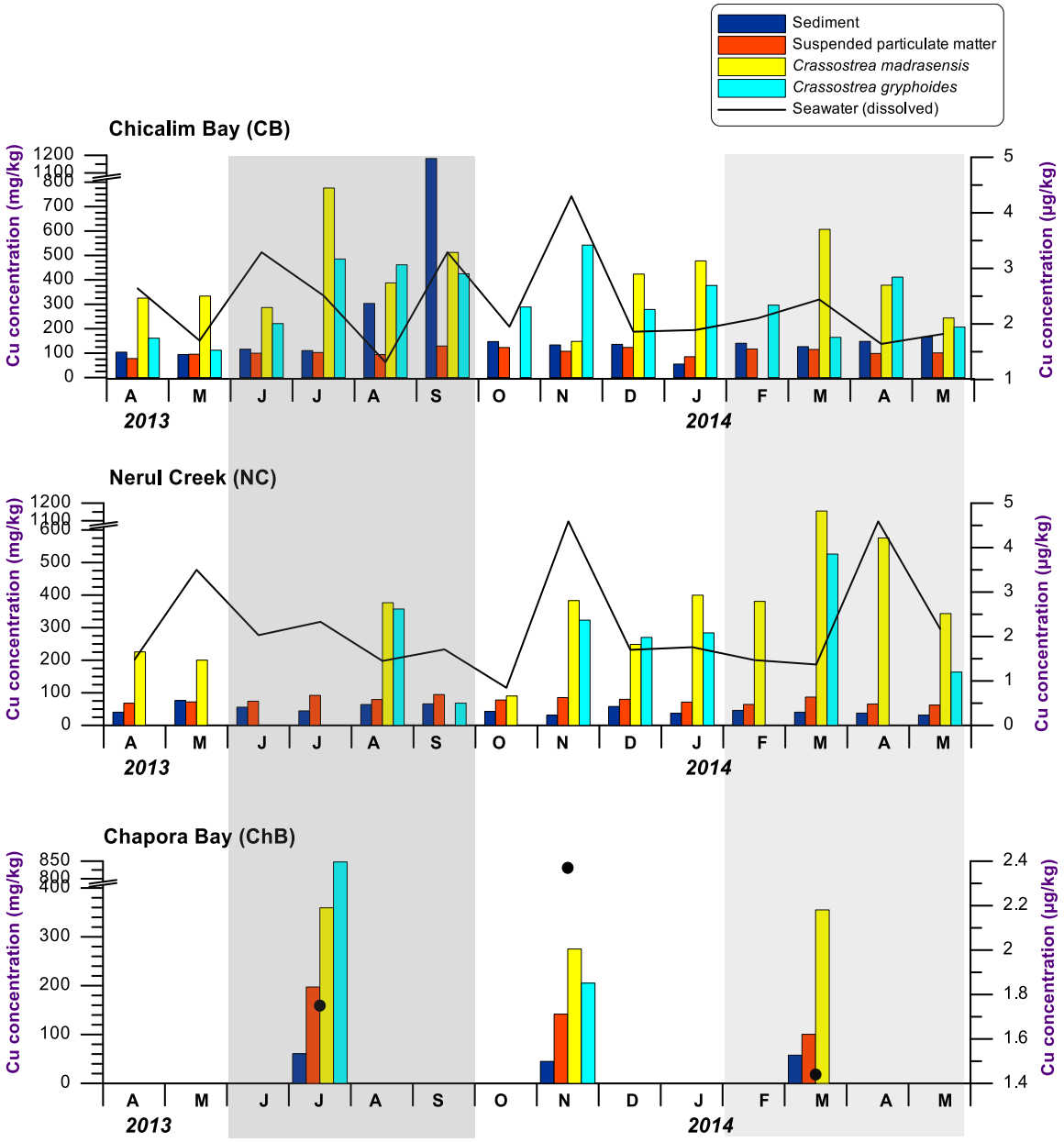

Figure 4.1 Concentration of Cu in surface sediment, SPM (suspended particulate matter), oyster (*Crassostrea madrasensis* and *Crassostrea gryphoides*) tissue (mg/kg) and surface seawater (dissolved) (µg/l) measured at three study sites during April 2013–May 2014. Values are presented as an average (n = 3).

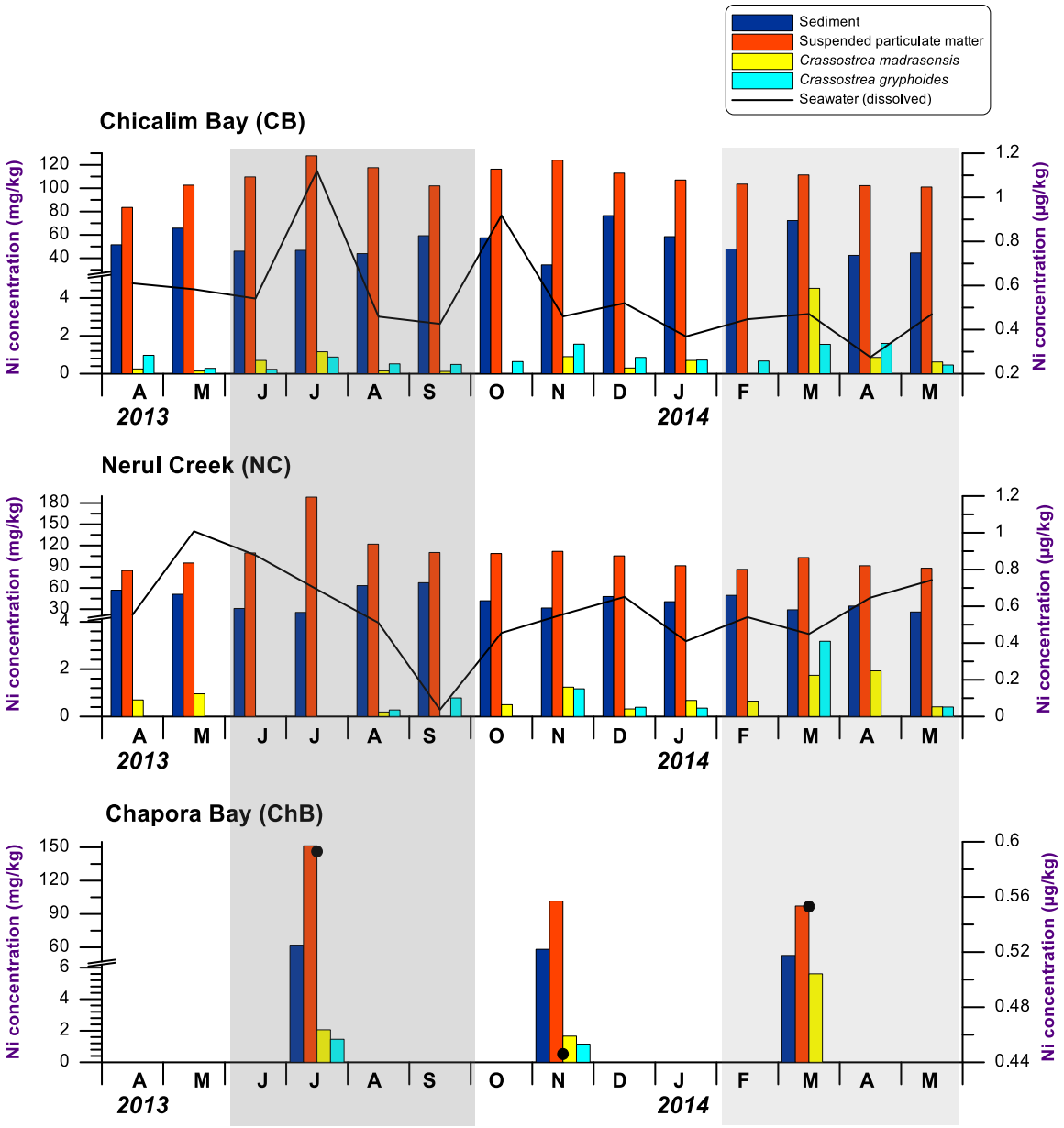

Figure 4.2 Concentration of Ni in surface sediment, SPM (suspended particulate matter), oyster (*Crassostrea madrasensis* and *Crassostrea gryphoides*) tissue, (mg/kg) and surface seawater (dissolved) (µg/l) measured at three study sites during April 2013–May 2014. Values are presented as an average (n = 3).

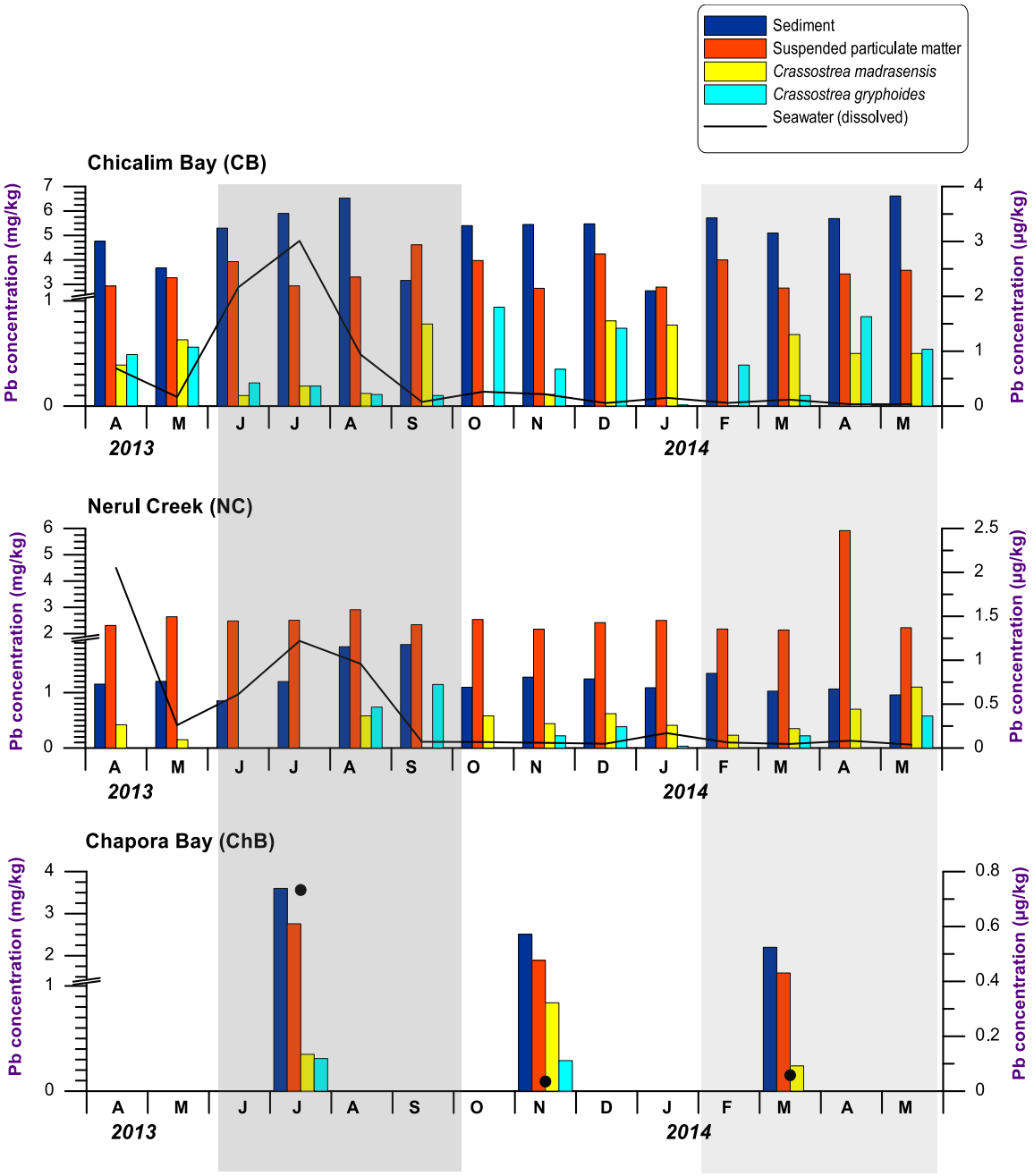

Figure 4.3 Concentration of Pb in surface sediment, SPM (suspended particulate matter), oyster (*Crassostrea madrasensis* and *Crassostrea gryphoides*) tissue, (mg/kg) and surface seawater (dissolved) (µg/l) measured at three study sites during April 2013–May 2014. Values are presented as an average (n = 3).

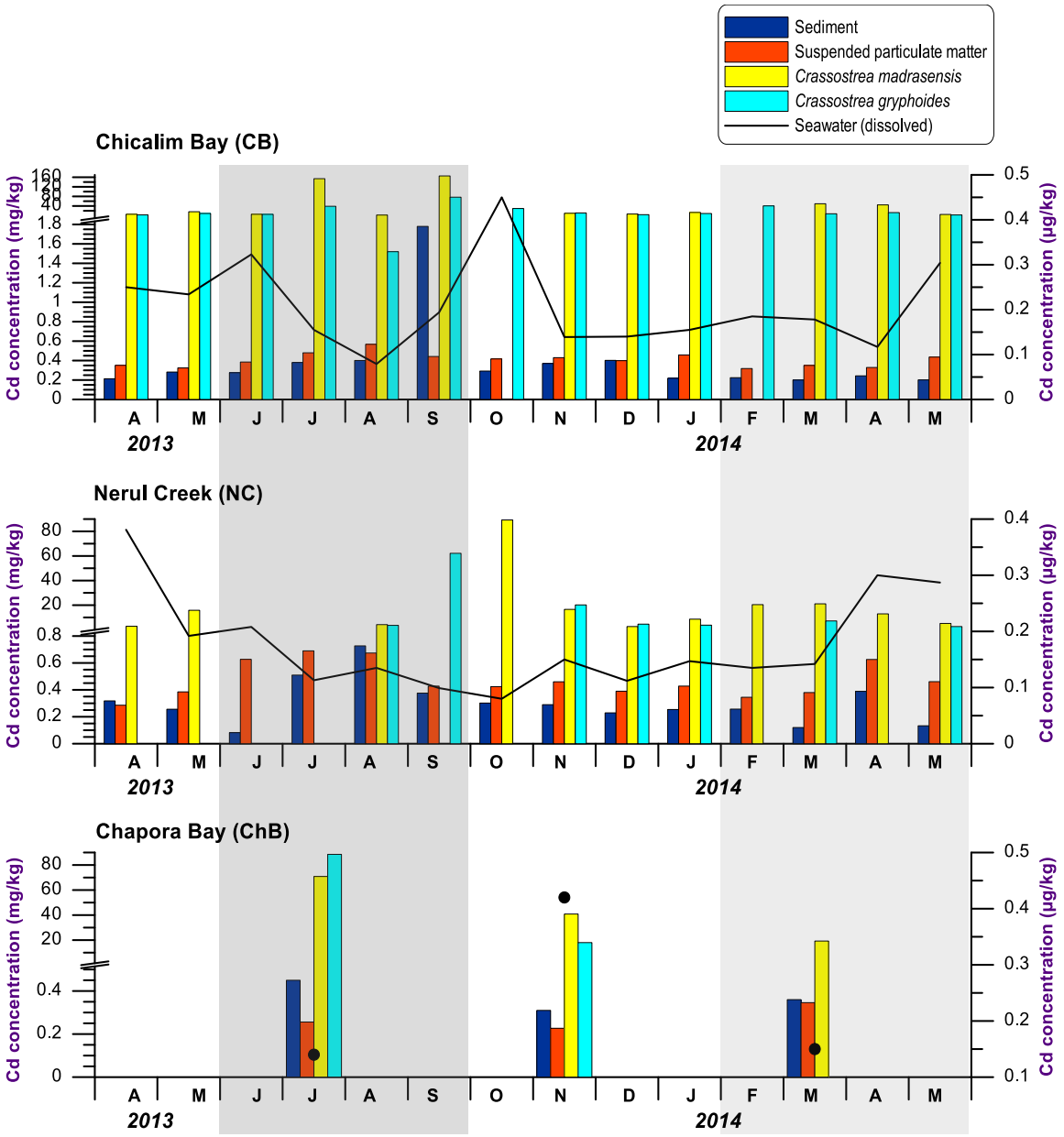

Figure 4.4 Concentration of Cd in surface sediment, SPM (suspended particulate matter) oyster (*Crassostrea madrasensis* and *Crassostrea gryphoides*) tissue, (mg/kg) and surface seawater (dissolved) (µg/l) measured at three study sites during April 2013–May 2014. Values are presented as an average (n = 3).

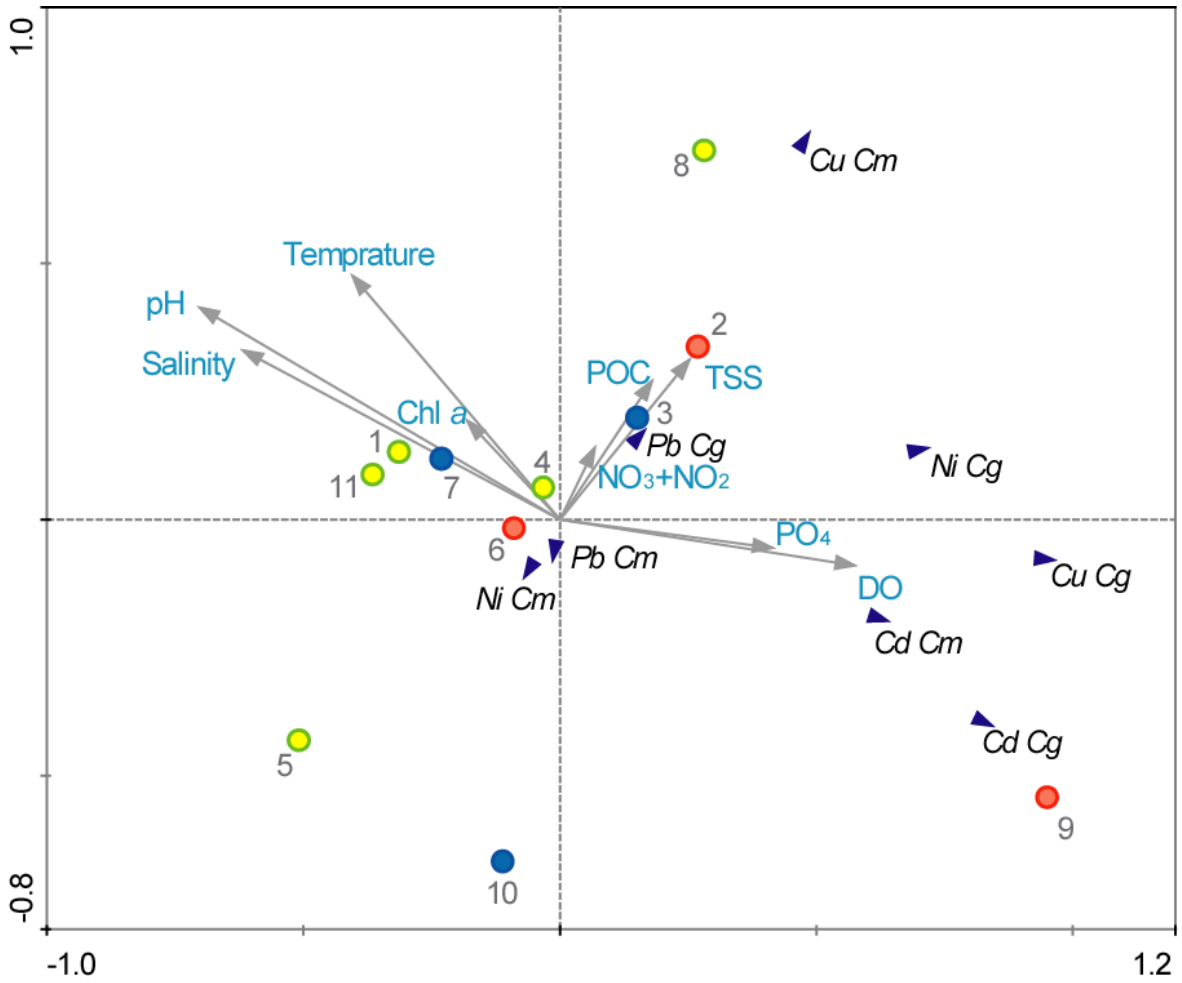

Figure 4.5 Redundancy analysis ordination diagram with metals concentration in tissue of oyster species in relation to water variables measured during four different seasons at study sites. Abbreviations: Cm = *Crassostrea madrasensis*, Cg = *Crassostrea gryphoides*, DO = dissolve oxygen (mg/l), Chl *a* = chlorophyll *a* (µg/l), NO_3-N = nitrate (µmol/l), NO_2-N = nitrite (µmol/l), PO_4-P = phosphate (µmol/l), POC = particulate organic carbon (µg C/l), TSS = total suspended solids (mg/l), CB = Chicalim Bay, NC = Nerul Creek, ChB = Chapora Bay, PreM1 = pre-monsoon 1, Mon = monsoon, PostM = post-monsoon, PreM2 = pre-monsoon 2. Metals concentration in oyster (*C. madrasensis* and *C. gryphoides*) tissue expressed in mg/kg. Numbers represents the samples such as 1: CB PreM1, 2: CB Mon, 3: CB PostM, 4: CB PreM2, 5: NC PreM1, 6: NC Mon, 7: NC PostM, 8: NC PreM2, 9: ChB Mon, 10: ChB PostM, 11: ChB PreM, yellow circle: pre-monsoonal samples, red circle: monsoonal samples, blue circle: post-monsoonal samples. Solid arrows represents the environmental variable, length of the arrows represents the relative importance of that variable, and triangle represents the metal concentration in oyster species.

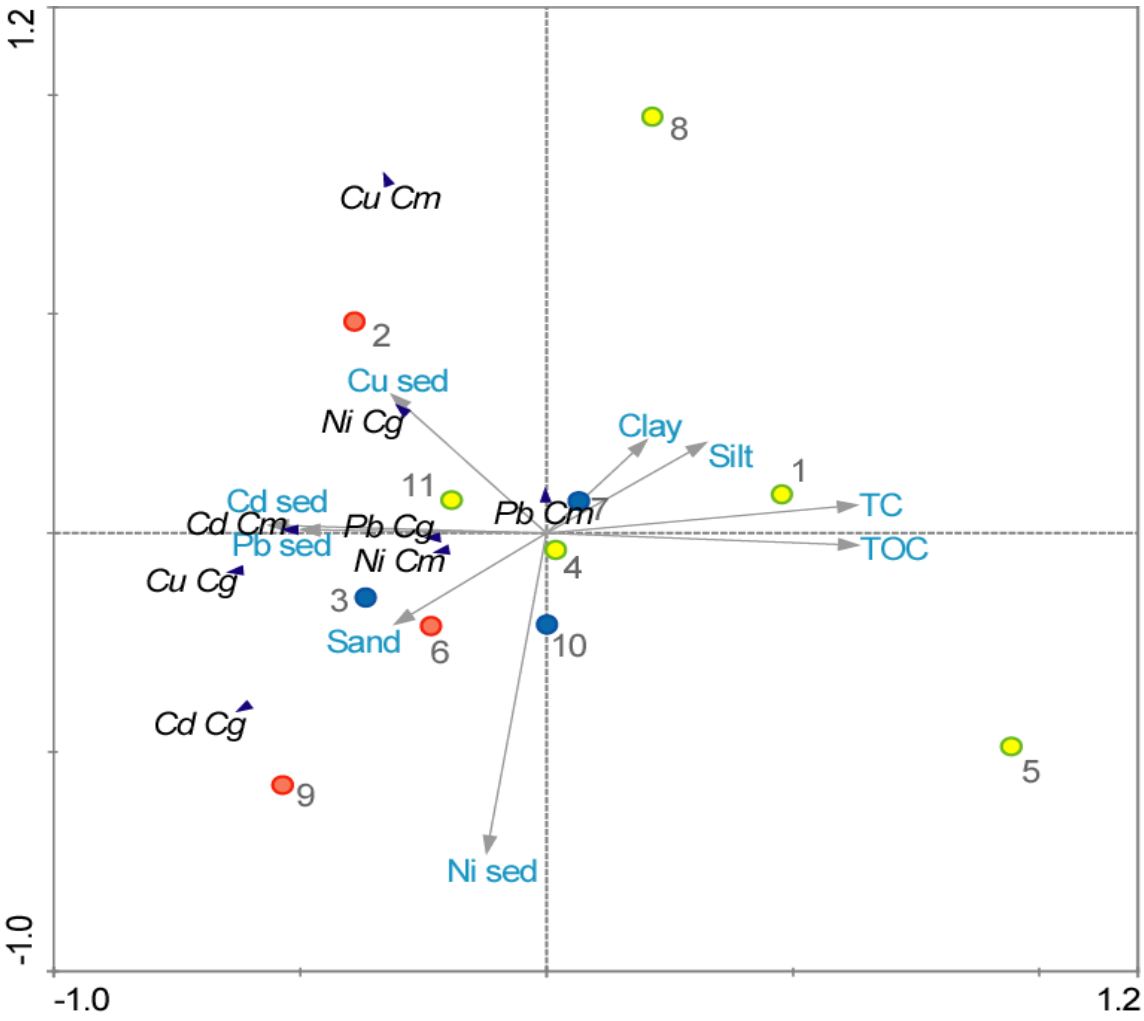

Figure 4.6 Redundancy analysis ordination diagram with metals concentration in tissue of oyster species in relation to sedimentary variables including metals concentration in surface sediment during four different seasons at study sites. Abbreviations: Cm = *Crassostrea madrasensis*, Cg = *Crassostrea gryphoides*, sed = sediment, TC = total carbon (%), TOC = total organic carbon (%), sand silt clay (%), CB = Chicalim Bay, NC = Nerul Creek, ChB Chapora Bay, PreM1 = pre-monsoon 1, Mon = monsoon, PostM = post-monsoon, PreM2 = pre-monsoon 2. Metals concentration in sediment, and oyster (*C. madrasensis* and *C. gryphoides*) tissue expressed in mg/kg. Numbers represents the samples such as 1: CB PreM1, 2: CB Mon, 3: CB PostM, 4: CB PreM2, 5: NC PreM1, 6: NC Mon, 7: NC PostM, 8: NC PreM2, 9: ChB Mon, 10: ChB PostM, 11: ChB PreM, yellow circle: pre-monsoonal samples, red circle: monsoonal samples, blue circle: post-monsoonal samples. Solid arrows represents the environmental variable, length of the arrows represents the relative importance of that variable, and triangle represents the metal concentration in oyster species.

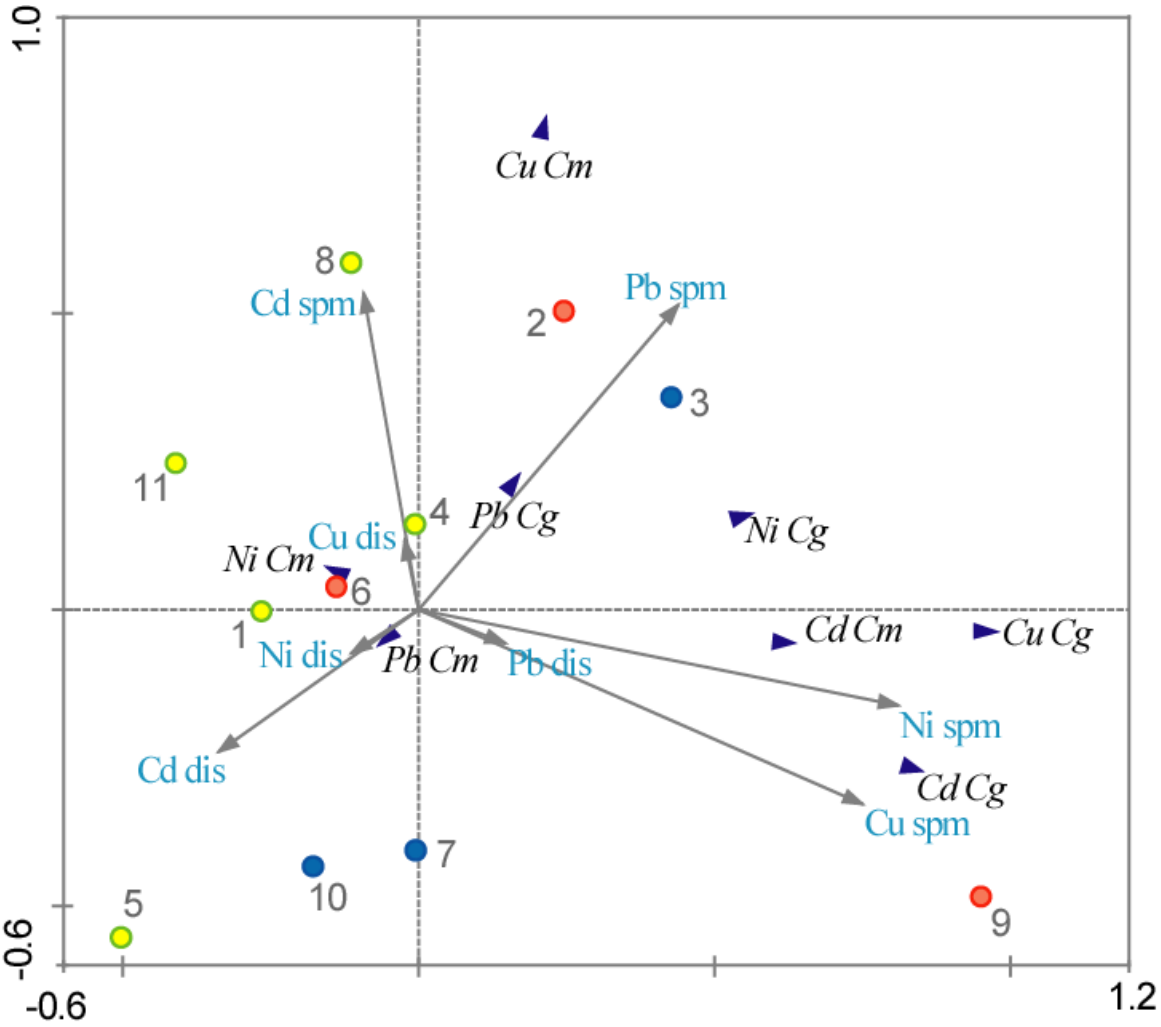

Figure 4.7 Redundancy analysis ordination diagram with metals concentration in tissue of oyster species in relation to dissolve and particulate metals concentration in surface seawater measured during four different seasons at study sites. Abbreviations: Cm = *Crassostrea madrasensis*, Cg = *Crassostrea gryphoides*; SPM = suspended particulate matter, dis = dissolve, CB = Chicalim Bay, NC = Nerul Creek, ChB = Chapora Bay, PreM1 = pre-monsoon 1, Mon = monsoon, PostM = post-monsoon, PreM2 = pre-monsoon 2. Metals concentration in SPM and oyster (*C. madrasensis* and *C. gryphoides*) tissue expressed in mg/kg, whereas in seawater (dissolved) expressed in µg/l. Numbers represents the samples such as 1: CB PreM1, 2: CB Mon, 3: CB PostM, 4: CB PreM2, 5: NC PreM1, 6: NC Mon, 7: NC PostM, 8: NC PreM2, 9: ChB Mon, 10: ChB PostM, 11: ChB PreM, yellow circle: pre-monsoonal samples, red circle: monsoonal samples, blue circle: post-monsoonal samples. Solid arrows represents the environmental variable, length of the arrows represents the relative importance of that variable, and triangle represents the metal concentration in oyster species.

Chapter 5

Effects of Dissolved Lead Concentrations on Oyster *Crassostrea madrasensis* (Preston, 1916)

5.1 Introduction

Lead (Pb) is ubiquitous in presence and one of the most toxic element occurs in almost all the ecosystem (Hsu and Guo, 2002; Flora et al., 2012). It is derived from both natural sources such as weathered (dissolved) or eroded (particulate) earth's surface, volcanic activities, atmospheric aerosol, biogenic forest fires as well as anthropogenic activities. In recent years, however, increase in various anthropogenic activities such as lead smelting, use of lead based-antifouling paints, ceramics, plastics, leaded gasoline, combustion of fossil fuel have significantly increased the Pb concentration in the aquatic environment (MacFarlane, 2001; Flora et al., 2012). High to moderate levels of Pb content have been found in tissues of edible clam *Paphia malabarica* (average: 30.3 mg/kg) (Krishna Kumari et al., 2006), suspended particulate matter (range: 27.6–91.4 mg/kg) (Kessarkar et al., 2013) and in surface sediments (average: 22.6 mg/kg) (Veerasingam et al., 2015) across the estuarine waters of Goa. Moreover, higher concentrations of Pb level (>1 mg/kg, permissible limit) have been observed in the tissues of edible oyster *Crassostrea* sp. from the coastal waters of Goa (Shenai-Tirodkar et al., 2016). It is recognized that impact of these accumulated metals cannot be judged only by quantification because it does not provide a clear indication of toxic effects of pollutant on the aquatic organism (Livingstone, 2001).

Biochemical biomarkers have been commonly used to measure the effects of contaminants on organisms in ecotoxicological studies (Livingstone, 1993). Under normal metabolic processes, a balance between generation and neutralization of reactive oxygen species (ROS) include superoxide anion radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2)

and hydroxyl radical (˙OH) is maintained by organisms. However, it has been proved that bioaccumulated metal ions enhance the formation of reactive radicals in the cells which cause the cell damage (Leonard et al., 2004; Valko et al., 2005). It is well known that exposure to Pb boosts the ROS production, which in turn results in damage to the cell membrane, protein oxidation, lipid peroxidation and DNA damage (Halliwell and Gutteridge, 1989; Gurer and Ercal, 2000; Ercal et al., 2001; Hsu and Guo, 2002; Flora et al., 2012) (Figure 5.1). This oxidative damage can be assessed by measuring thiobarbituric acid reactive substances (TBARS) reflecting the state of lipid peroxidation (LPO) of the cell membrane (Ohkawa et al., 1979). Further, to protect against oxidative stress organism possess major antioxidant defense system which includes three main enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), and glutathione-s-transferase (GST). However, noticeable variation in responses (induction and/or inhibition) of biochemical biomarker were recorded in previous laboratory and field studies which mostly depend on the type and amount of contaminants, type of species, the tissue, and the sampling time (Zanette et al., 2006; Luna-Acosta et al., 2010; Cotou et al., 2013).

Presently, there is very limited work published with respect to Pb exposure in bivalves (Prakash and Jagannatha Rao, 1995; Yan et al., 1997; Dafre et al., 2004; Fernandez et al., 2010). Particularly, Pb toxicity study on oyster species from Indian coast has not been conducted which is harvested and consumed on large scale and on regular basis. Exposure of oysters to various concentrations of Pb, coming from discharge of dissolved and particulate forms of Pb into the marine ecosystem of Goa coast (India), has

necessitated this study to understand the antioxidant defense response of oysters against Pb exposure. It has been proved that field-based effects could well be understood by conducting laboratory exposures of organisms to a particular contaminant (Verlecar et al., 2008).

In this study, gills and digestive glands of oyster, *Crassostrea madrasensis*, were examined to assess the effect of Pb exposure using biochemical markers such as LPO, SOD, CAT and GST. The reason for selecting gills and digestive gland is because these tissues form an active site for xenobiotic uptake and oxy-radical generation as well as enzyme biotransformation process (Livingstone et al., 1992).

5.2 Materials and methods

5.2.1 Sampling Site and Oyster Collection

A total of 150 individual adult oysters, *Crassostrea madrasensis* (6–7 cm), irrespective of sex, were collected during low tide in post-monsoon (October 2015) from Galgibag estuary, South Goa coast, India. Galgibag site (Lat. 14° 57' 54.66" N, Long. 74° 03' 20.16" E) was chosen because this site is located far away from potential pollution sources such as industries and harbor activities (Figure 5.2, Plate 5.1). The hydrological parameters (water temperature: 29 ± 1 °C, pH: 7.9±0.4 and salinity: 27 ± 1) were recorded at the time of oyster collection. The samples were brought to laboratory in seawater and cleaned to remove fouling organisms and debris. All the oysters were acclimatized for 8 days in aerated seawater at room temperature before conducting the acute semi-static renewal Pb toxicity (8 days) experiment (Thompson et al., 2012).

5.2.2 Experimental Design

After the acclimatization period, the oysters were distributed into five different groups (n = 15, each tank) and subjected to five treatments: one seawater control and four different Pb concentrations (1 µg/l, 10 µg/l, 25 µg/l, and 50 µg/l) (Plate 5.2). These concentrations were selected based on environmental realistic concentration (Shenai-Tirodkar et al., 2016) and concentration large enough to induce biomarker response. Mortality was monitored and dead oysters were removed. Under these conditions, the state of the antioxidant enzyme system was assessed.

The experiment was setup in duplicate in 25 l capacity plastic tanks (~1.5 l of seawater per oyster). Seawater was continuously aerated and standard hydrological parameters (temperature 28 ± 1 °C, pH 8.2 ± 0.4 and salinity 27 ± 1) were recorded throughout the experiment. Lead nitrate [Pb $(NO_3)_2$] (Merck India Ltd, Mumbai) was used to prepare stock solution (100 mg/l) and preserved in a refrigerator until use. This was treated as main stock solution from which fresh sub-stock solutions were prepared and used for preparation of required concentrations of Pb. All the glasswares used during experiment were washed with 10 % HNO_3 (nitric acid) and well rinsed with distilled water. The whole exposure media (10 l) was renewed on daily basis and replaced with fresh natural seawater, maintaining respective concentrations of Pb $(NO_3)_2$ such as 1 µg/l, 10 µg/l, 25 µg/l, and 50 µg/l by adding 0.1ml, 1ml, 2.5 ml and 5 ml of stock solution (100 mg/l) of Pb $(NO_3)_2$, respectively. Oysters were not fed during experimentation. At each sampling (2nd, 4th, 6th and 8th day of experiment duration), two of the surviving oysters were sacrificed from each treatment tank and immediately gills and digestive gland were

dissected for further analysis. All the further analyses were performed in triplicates to rule out the experimental bias.

5.2.3 Determination of Pb Content in Gills and Digestive Gland of C. madrasensis

Dissected gills and digestive gland of *C. madrasensis* were processed for Pb estimation according to the method described in Chapter 4 section 4.2.2.5. Determination of Pb was made by using Flame Atomic Absorption Spectrometer (Flame-AAS). Utmost care was taken at every step of processing the sample, to avoid contamination. All reagents used in the analysis were of suprapure grade. Appropriate blanks and standards were also prepared by using the same method. Appropriate dilutions of samples were made depending on the sensitivity of detection in these samples. Precision measured as relative standard deviation (% RSD) of triplicate sample values were <10 %. Accuracy of Pb analysis was expressed in recovery percentage 91.35 %.

5.2.4 Biochemical Analysis in Gills and Digestive Gland of C. madrasensis

5.2.4.1 Sample Preparation

The gills and digestive gland of *C. madrasensis* were thoroughly washed with phosphate buffer (50 mM; pH 7.4) and homogenized in 50 mM phosphate buffer (1 mM EDTA, 1mM DTT, 0.15 M KCl, 0.01 % PMSF, pH 7.4). Homogenization was carried out using motor driven Teflon Potter-Elvejhen homogenizer. Further, homogenates were centrifuged at 10000 × g for 30 minutes at 4 °C. The resulting supernatant fractions were stored at -80 °C until the quantification of the activity of LPO, SOD, CAT and GST. All the further biochemical analyses were performed in triplicates.

5.2.4.2 Estimation of Lipid Peroxidation (LPO)

The activity of LPO was estimated using the protocol described by Ohkawa et al. (1979) with minor modifications. Briefly, tissue homogenate (0.5 ml) was mixed with 1.5 ml of 0.8 % TBA solution, 1.5 ml of 20 % acetic acid (pH 3.5), 0.2 ml of 8.1 % SDS, 0.2 ml double distilled water and 0.1 ml of 0.76 % BHT. The mixture was heated at 95 °C for 60 minutes and cooled at room temperature. After that samples were centrifuged at 5000 × g for 10 minutes and the absorbance of the supernatant was read at 532 nm against blank. The LPO index was calculated by using an extinction coefficient of 1.56×105/M/cm (Wills, 1969) and expressed as nmol TBARS formed/mg protein.

5.2.4.3 Estimation of Superoxide Dismutase (SOD) and Catalase (CAT) Activity

Superoxide dismutase (EC 1.15.1.1) and catalase (EC 1.11.1.6) activities were measured by using EnzyChrom superoxide dismutase assay kit (ESOD-100) and EnzyChrom catalase assay kit (ECAT-100) respectively as per the manufacturer's instructions. Both the kits were bought from Bioassay Systems, USA. Assays were performed in a microplate reader FLUOstar Omega, BMG LABTECH Pty. Ltd.) by using 96 well plates. The results of SOD were expressed as Unit/mg protein whereas CAT activity was presented as nkat/mg protein.

5.2.4.4 Estimation of Glutathione S-Transferase (GST) Activity

Glutathione S-Transferase (EC 2.5.1.18) activity was determined following the method of Mannervik (1985) with minor modifications. The reaction mixture contained 950 µl

assay buffer PBS (pH 6.5), 10 µl of 100 mM GSH, 30 µl sample (200-250 µg protein) and 10 µl of 100 mM CDNB. The change in absorbance was recorded at 340 nm for every 30 seconds for 5 minutes. The activity of GST was determined using molar extinction coefficient of 0.0096/µM/cm for CDNB. The results were expressed as nmol CDNB conjugates formed/min/mg protein.

5.2.4.5 Estimation of total Protein Concentration

Total protein concentrations of tissue samples were analyzed according to the Lowry method (Lowry et al., 1951). In brief, to each tube containing 0.1 ml of suitably diluted sample, 0.4 ml homogenizing buffer was added followed by 5 ml of biuret reagent and the test tubes were allowed to stand for 10 min at room temperature. To the reaction mixture, 0.5 ml of Folin-Ciocalteu phenol reagent (1:2 v/v) diluted with distilled water was added and mixed well. The tubes were kept at room temperature for 30 minutes. The colour intensity was measured against the suitable blank at 700 nm using spectrophotometer. Bovine serum albumin was used to prepare standard curve.

5.2.4.5.1 Preparation of Biuret Reagent:

Solution A: 2 % (W/V) sodium carbonate (Na_2CO_3) in 0.1 N sodium hydroxide (NaOH) solution.

Solution B: 0.5 % (W/V) copper sulphate ($CuSO_4$) in distilled water.

Solution C: 1 % (W/V) potassium-sodium tartarate ($KNaC_4H_4O_6$) in distilled water.

Working biuret solution: 100 ml of solution A + 2 ml of solution B + 2 ml of solution C

5.2.5 Statistical Analysis

Data were expressed as average ± standard deviation (SD) (n = 3). All the data were first checked for normality using Shapiro–Wilk's test, and homogeneity of variance by using Levene's test. Data that failed to meet the normal distribution were transformed by log (x+1) transformation prior to statistical testing. Significance of variability among groups with Pb concentrations, exposure time and the types of tissue were evaluated for each biomarker as well as for the Pb content by using three-way analysis of variance (three-way ANOVA). For the multiple comparison among groups post hoc test (Tukey's HSD test) was performed. To find out the significant relationship, Pearson correlations were performed among Pb content, oxidative stress and antioxidant response in gills and digestive glands of *C. madrasensis*. Statistical significance of data was measured at $P<0.05$ level. All the tests were performed using STATISTICA 8 (StatSoft, Inc., USA) software.

5.3 Results

During acclimatization period, dead oysters were removed from the respective tanks. After acclimatization, during experimental period oyster's mortality was observed only in tanks containing concentration of Pb more than 10 µg Pb/l. A total of 3 oyster died from 10 µg Pb/l treated tank on 5th and 7th day. Similarly, 3–4 oysters died at 25 µg Pb/l and 50 µg Pb/l treated tank on 4th and 7th day respectively.

5.3.1 Concentration of Pb in Gills and Digestive Gland of C. madrasensis

Concentrations of Pb in gills ranged from 0.27 ± 0.07 mg/kg to 1.27 ± 0.01 mg/kg whereas in digestive gland, values varied between 0.1 ± 0.05 mg/kg and 0.95 ± 0.05 mg/kg (Figure 5.3). On the 8th day, 50 µg/l treated oysters showed the highest accumulation (1.27 ± 0.01 mg/kg) in gills which is more than the allowable limit for human consumption according to guidelines set by EU, 2001. Accumulation of Pb in the gills and digestive gland of *C. madrasensis* increased significantly ($p<0.05$) over a period of days in each Pb treatments as compared to control. Further, significantly higher accumulation of Pb was observed in gills than digestive gland during the experiment at all the exposed concentrations (Table 5.1).

5.3.2 Biomarker Responses in Gills and Digestive Glands of C. madrasensis

Oxidative stress indices such as LPO, SOD, CAT, and GST in gills and digestive gland of oyster were measured under the semi-static Pb exposure experiment. Considerable changes took place in the oxidative defense system of both gills and digestive gland after exposure to different concentration of Pb. Results of three-way ANOVA revealed the significant ($p<0.05$) differences in biomarker responses among variables such as concentrations, exposure time (days), types of tissue and interactions among these variables (Table 5.2).

5.3.2.1 Lipid Peroxidation (LPO)

LPO index in both gills and digestive gland showed significant ($p<0.001$) increase against each Pb treatment as compared to respective control. Moreover, LPO index

showed strong positive correlation with the Pb content (p<0.001) in gills and digestive gland (Table 5.3). Digestive gland exhibited significantly higher oxidative stress compared to gills on 2nd and 6th day (Figures 5.4 a and b). Further, the digestive gland of oysters exposed to 50 µg/l showed declined LPO activity significantly from 6th day to 8th day (p<0.001).

5.3.2.2 Superoxide Dismutase (SOD)

Figures (5.4 c and d) and Table 5.2 shows that SOD activity in digestive gland was significantly higher than gills. SOD activity in gills and digestive gland from control tank did not show much variation during experimental successive exposure periods. In particular, gills showed significant (p<0.001) increase in SOD activity at 10, 25 and 50 µg/l treated groups until 6th day, which then dropped prominently (p<0.001) on 8th day (except at 10 µg/l). A similar trend was observed in digestive gland from all the treated groups (except in 50 µg/l treated group where SOD activity significantly increased). Activities of SOD was closely correlated with Pb concentrations and LPO index in both the tissues (Table 5.3).

5.3.2.3 Catalase (CAT)

Activity of CAT in gills increased significantly (p<0.001) until the 4th day in 1 and 10 µg Pb/l exposed groups and till the 6th day in 25 and 50 µg/l exposed groups. This was followed by a slight decrease in CAT values on 8th day. Whereas, oyster gills from control group showed no significant variation in CAT measurements throughout the experiment. A similar trend of CAT activities was also noticed in digestive gland of

treated oysters (p<0.001) (Figures 5.4 e and f). The CAT activity showed a positive correlation with Pb concentration, LPO (p<0.01) and GST (p<0.05) in both gills and digestive gland of oyster (Table 5.3).

5.3.2.4 Glutathione S-Transferase (GST)

Response of GST in gills and digestive gland to Pb exposure are shown in Figures 5.4 g and h. Low levels of GST were observed in gills and digestive gland (p<0.05) in all Pb exposed groups compared to controls till the 6th day (except in 50 µg/l). However, on the 8th day, GST levels in gills increased in 10, 25 and 50 µg/l exposed groups while in digestive gland it increased in all the exposed groups (1, 10, 25 and 50 µg/l). Activities of GST in gills was positively correlated with Pb concentration, LPO and CAT whereas Pb concentration, LPO and SOD showed positive correlation in digestive gland (Table 5.3).

5.4 Discussion

Under metal exposure, oyster accumulates metal at very high concentrations in different body parts to different extents (Marigomez et al., 2002) which generates several biological responses depending on metal concentration and duration of exposure (Hariharan et al., 2014). In the present experimental set up mortality of oysters were observed at the higher Pb concentrations (10, 25 and 50 µg/l). This could be due to increase in the levels of intracellular toxic metabolites. Moreover, accumulations of Pb in live oyster's gills was found to be approximately 2–4 times higher than in the digestive gland. The reasons for the higher concentrations in gills may be due to its continuous exposure to the surroundings during filtration process and the large surface area of thin

epithelium (Soldatov et al., 2007; Fernandez et al., 2010). Similar to these results, Prakash and Jagannatha Rao (1995) also recorded high metal accumulation in gills than digestive gland of mussel *Perna viridis*. Alcutt and Pinto (1994) observed Pb equilibrium between tissue of clam, *Mercenaria mercenaria* and surrounding aquatic medium within short exposure (five days). The high accumulation of Pb in tissues of bivalves occurs due to the low excretion rates of amorphous granules and their immobilization in the shell (Viarengo, 1989).

In the current study, TBARS product was prominently increased in gills and digestive gland in all Pb exposed groups compared to unexposed (control) group. Significant positive correlation ($p<0.001$) was found between LPO and Pb concentration in both the tissues (Table 5.3). This infers that oxidative stress increases as a consequences of rise in ROS formation in lipid membranes due to gradual increase in Pb accumulation (Stohs and Bagchi, 1995; Ercal et al., 2001). It is known that marine organisms that are rich in polyunsaturated fatty acids increase ROS generation during metal exposure (Liu et al., 1997). This can lead to an imbalance in pro-oxidant and antioxidant processes, enhance oxidative damage and generate TBARS product (Manduzio et al., 2005). Another reason for high level of TBARS induction in both the tissues from exposed groups compared to control group may be due to the structural and functional characteristics of both the organs. Firstly, it is important to point out that continuous oxidative load was experienced by gills in this study. As discussed earlier, an oyster's gill structure may be the reason that it showed more effect of Pb stress in successive concentrations (Soldatov et al., 2007). Secondly, since the digestive gland is the main organ involved in metabolic

function, stress induced from various Pb concentrations may directly affect the metabolic pathway, resulting in both increased production and accumulation of ROS (Livingstone et al., 1992; Trombini et al., 2010). Consequences of these generated free radicals damages the membrane phospholipids and also change in fatty acid composition of molecules (Knowles and Donaldson, 1990; Hsu and Guo, 2002). This oxidative stress can further bring changes in the antioxidant enzyme activities (Manduzio et al., 2005; Gonzalez et al., 2015). The results of present study are corroborated by the findings of previous studies where researchers have found increased LPO due to metal toxicity in mussels (Prakash and Jagannatha Rao, 1995; de Almeida et al., 2004; Vlahogianni and Valavanidis, 2007; Tsangaris et al., 2010) and prawn *Penaeus monodon* (Hariharan et al., 2012).

Superoxide dismutase is the first and crucial antioxidant enzyme which removes the superoxide radical through the process of dismutation to oxygen and hydrogen peroxide ($2O_2^{\bullet-} + H^+ \rightarrow H_2O_2 + O_2$) (Fridovich, 1975). It prevents generation of highly toxic $^{\bullet}OH$ radicals. In the present study, SOD activity was slightly more in the digestive gland than gills. Moreover, positive correlation ($p<0.001$) was found between the SOD and LPO with Pb concentration in both the tissues (Table 5.3). The observed positive correlation indicates that response of SOD was linked to Pb accumulation and consequently oxidative stress. During experimental period, SOD increased noticeably until the 6th day of exposure that decreased slightly on the 8th day in both the organs (prominently in gills). The initial increase in SOD suggests the protective behavior of the cell against $O_2^{\bullet-}$ radicals produced by activation of the SOD initially while its depletion later indicates the

probable SOD degradation by ROS or it's over utilization to overcome Pb toxicity (Dafre et al., 2004). Furthermore, SOD activities in gills dropped more rapidly than those of the digestive gland suggesting that the detoxification function of digestive gland is more effective than the gills to counteract the high LPO level generated under plausible Pb toxicity (Trombini et al., 2010). This results are in the accordance with previous studies carried out by Jing et al. (2007) on pearl oyster *Pinctada fucata* where SOD was decreased gradually in later part of Pb exposure experiment. Dai et al. (2012) were also recorded the inhibition of SOD in tilapia (*Oreochromis niloticus*) during dietary Pb exposure.

Catalase is a ubiquitous oxidoreductase antioxidant enzyme that breaks down the hydrogen peroxide molecule to water and oxygen molecules ($2H_2O_2 \rightarrow 2H_2O + O_2$) and thus removes the toxicity of H_2O_2 (Livingstone, 2001). In the present study, significant positive correlation ($p<0.01$) was detected between CAT and Pb concentrations, LPO, SOD which suggest that SOD and CAT function together in a coordinated manner and neutralizes the generation of H_2O_2, against the oxidative stress induced by Pb (Jena et al., 2009). Moreover, like SOD, CAT levels in both the tissues (gills and digestive gland) increased until the 4th and 6th day and declined slightly in the later phase of the experiment. These reductions in CAT impairs scavenging of H_2O_2 radical leading to the enhancement of LPO in both the tissues (Ercal et al., 2001). Similar results were obtained in green mussel *P. viridis* under Pb exposure (Hariharan et al., 2014). According to our results, low activities of SOD and CAT indicate a non-significant contribution of these enzymes to antioxidant defense processes in the later phase of the experiment, which

further may be associated with difficulty in compensating oxidative stress. Similarly, it has been observed that CAT and SOD target Pb by forming the complexes with their substrates which is responsible for inhibition/deactivation of these enzymes (Ercal et al., 2001; Marques et al., 2016). This decrease in SOD and CAT may results in increase in $O_2^{\bullet-}$ radicals accumulation with combination of H_2O_2 generate hydroxyl $^{\bullet}OH$ radicals (under classic Haber–Weiss reaction) resulting in rise of TBARS substances (Verlecar et al., 2008).

Glutathione-S-Transferase is a major phase II-metabolic enzyme which plays a major role in detoxification of both xenobiotics and oxidative metabolic by-products (Van der Oost et al., 2003; Jozefczak et al., 2012). In the present study, gills and digestive gland showed initially less GST response which was below control level but it increased progressively from the 6th day onwards, indicating activation of detoxification mechanism in the gills as well as digestive gland cells. Apparently, the GST did not play an important role in neutralization of oxidative load initially, as judged by the low level of GST. Moreover, compensatory adaptive mechanisms between different biochemical biomarkers may occur (Regoli and Principato, 1995). Significant increase in GST activities in the later phase (8th day) of the experiment demonstrates the protective action of GST against reactive oxygen radicals to reduce Pb toxicity. This is also supported by the Pearson's correlation results where GST is positively correlated with the Pb concentration (p<0.001), LPO (p<0.01). This increase in GST activities may be a compensatory adaptive mechanism to neutralize increased levels of ROS when SOD and CAT were depleted from the 4th day onwards (Fernandez et al., 2010). Furthermore,

digestive gland showed more GST activities as compared to gills. This elucidates that the gills are weak in detoxification process in the oxidative defense system as mentioned earlier. These results also corroborate the findings of Yan et al. (1997) where the authors found an effective increase in the glutathione content in the gills and digestive gland of green mussel *P. viridis* after ~21 days of exposure to Pb stress. Similarly, Dafre et al. (2004) observed an increase in glutathione level against the Pb stress in brown mussel *Perna perna*. In contrast, some researchers have found that Pb inactivates glutathione by binding it to sulfhydryl group present in it, which further depresses the glutathione level (Ahamed and Siddiqui, 2007). Thus, measurement of a single antioxidant is not enough to state a complete antioxidant defense under laboratory as well as field conditions. On the whole, our study shows that Pb generate the oxidative stress in gills and digestive gland and induces activation of SOD and CAT in the initial phase, whereas GST takes a position as a compensatory of the defense system in later stages. In this experiment, Pb toxicity arises may be due to two different, although related pathways: first, the ROS formation which includes $O_2^{\bullet-}$, H_2O_2 and $^{\bullet}HO$ and second, the depletion of antioxidant reserves (Flora, 2002).

5.5 Conclusion

Based on the results of the investigated biochemical biomarkers LPO, SOD, CAT and GST it is concluded that oxidative stress generated in gills and digestive gland of *C. madrasensis* is specifically depend on the concentrations and duration of Pb exposure. Furthermore, acute to high levels (≥ 10 µg/l) of dissolved Pb in surrounding seawater could be harmful to the physiology of the oyster. However, in future, similar studies

should be conducted for longer exposure periods and with other biomarkers to understand in detail mechanisms of oxidative stress regulation in this sentinel species.

Table 5.1 Results of three-way ANOVA on the Pb concentrations in gills and digestive glands of *Crassostrea madrasensis* under control and [Pb $(NO_3)_2$] (1 µg/l, 10 µg/l, 25 µg/l, 50 µg/l) treated conditions during 8 days experiment.

Variables	SS	*Df*	MS	F	*p* value
Day	1.26266	3	0.42089	56.256	*0.000*
Concentration	4.82320	4	1.20580	161.167	*0.000*
Tissue	1.57094	1	1.57094	209.972	*0.000*
Days × Concentration	1.01723	12	0.08477	11.330	*0.000*
Days × Tissue	0.11394	3	0.03798	5.076	*0.003*
Concentration × Tissue	0.17656	4	0.04414	5.900	*0.000*
Days × Concentration × Tissue	0.16915	12	0.01410	1.884	*0.049*

Italic values denotes the significant level at $p<0.05$. Abbreviations: SS = sum of squares, Df = degrees of freedom, MS = mean sum of squares, F = F value. Days: 2nd, 4th, 6th, 8th day.

Table 5.2 Results of three-way ANOVA on the biomarker responses measured in gills and digestive glands of *Crassostrea madrasensis* under control and $PbCl_2$ (1 µg/l, 10 µg/l, 25 µg/l, 50 µg/l) treated conditions during 8 days experiment.

Biomarker	Factors	SS	*Df*	MS	F	*p* value
LPO	Day	0.41706	3	0.13902	1237.8	*0.000*
	Concentration	1.54713	4	0.38678	3443.8	*0.000*
	Tissue	11.71074	1	11.71074	104270.2	*0.000*
	Day × concentration	0.08256	12	0.00688	61.3	*0.000*
	Day × tissue	0.01834	3	0.00611	54.4	*0.000*
	Concentration × tissue	0.37175	4	0.09294	827.5	*0.000*
	Day × concentration × tissue	0.08289	12	0.00691	61.5	*0.000*
SOD	Day	63.72	3	21.24	13.016	*0.000*
	Concentration	887.94	4	221.99	136.046	*0.000*
	Tissue	11166.56	1	11166.56	6843.508	*0.000*
	Day × concentration	73.51	12	6.13	3.754	*0.000*
	Day × tissue	61.19	3	20.40	12.500	*0.000*
	Concentration × tissue	853.18	4	213.30	130.720	*0.000*
	Day × concentration × tissue	72.99	12	6.08	3.727	*0.000*
CAT	Day	2724	3	908	111.7	*0.000*
	Concentration	3330	4	832	102.4	*0.000*
	Tissue	88	1	88	10.8	*0.002*
	Day × concentration	4662	12	389	47.8	*0.000*
	Day × tissue	128	3	43	5.3	*0.002*
	Concentration × tissue	394	4	98	12.1	*0.000*
	Day × concentration × tissue	2222	12	185	22.8	*0.000*
GST	Day	0.6502	3	0.2167	106.1	*0.000*
	Concentration	0.4196	4	0.1049	51.4	*0.000*
	Tissue	0.1014	1	0.1014	49.7	*0.000*
	Day × concentration	0.6426	12	0.0535	26.2	*0.000*
	Day × tissue	0.0513	3	0.0171	8.4	*0.000*
	Concentration × tissue	0.6018	4	0.1504	73.6	*0.000*
	Day × concentration × tissue	1.0891	12	0.0908	44.4	0.000

Italic values denotes the significant level at $p<0.05$. Abbreviations: SS = sum of squares, Df = degrees of freedom, MS = mean sum of squares, F = F value. Days: 2nd, 4th, 6th, 8th day.

Table 5.3 Pearson correlation coefficient (r value) between Pb accumulation and various antioxidant parameters in gills and digestive gland from control and exposed *Crassostrea madrasensis.*

	Gills					Digestive gland				
Parameters	Pb content	LPO	SOD	CAT	GST	Pb content	LPO	SOD	CAT	GST
Pb content	1.00					1.00				
LPO	**0.91**^{c}	1.00				**0.91**^{c}	1.00			
SOD	**0.71**^{c}	**0.72**^{c}	1.00			**0.81**^{c}	**0.76**^{c}	1.00		
CAT	**0.59**^{b}	**0.58**^{b}	**0.53**^{a}	1.00		**0.67**^{b}	**0.68**^{b}	**0.61**^{b}	1.00	
GST	**0.77**^{c}	**0.68**^{b}	0.36	**0.47**^{a}	1.00	**0.79**^{c}	**0.70**^{b}	**0.53**^{a}	0.42	1.00

Significance level at $p<0.05$ = a, $p<0.01$ = b, $p<0.001$ = c. LPO = lipid peroxidation, SOD = superoxide dismutase, CAT = catalase, GST = glutathione-s-transferase

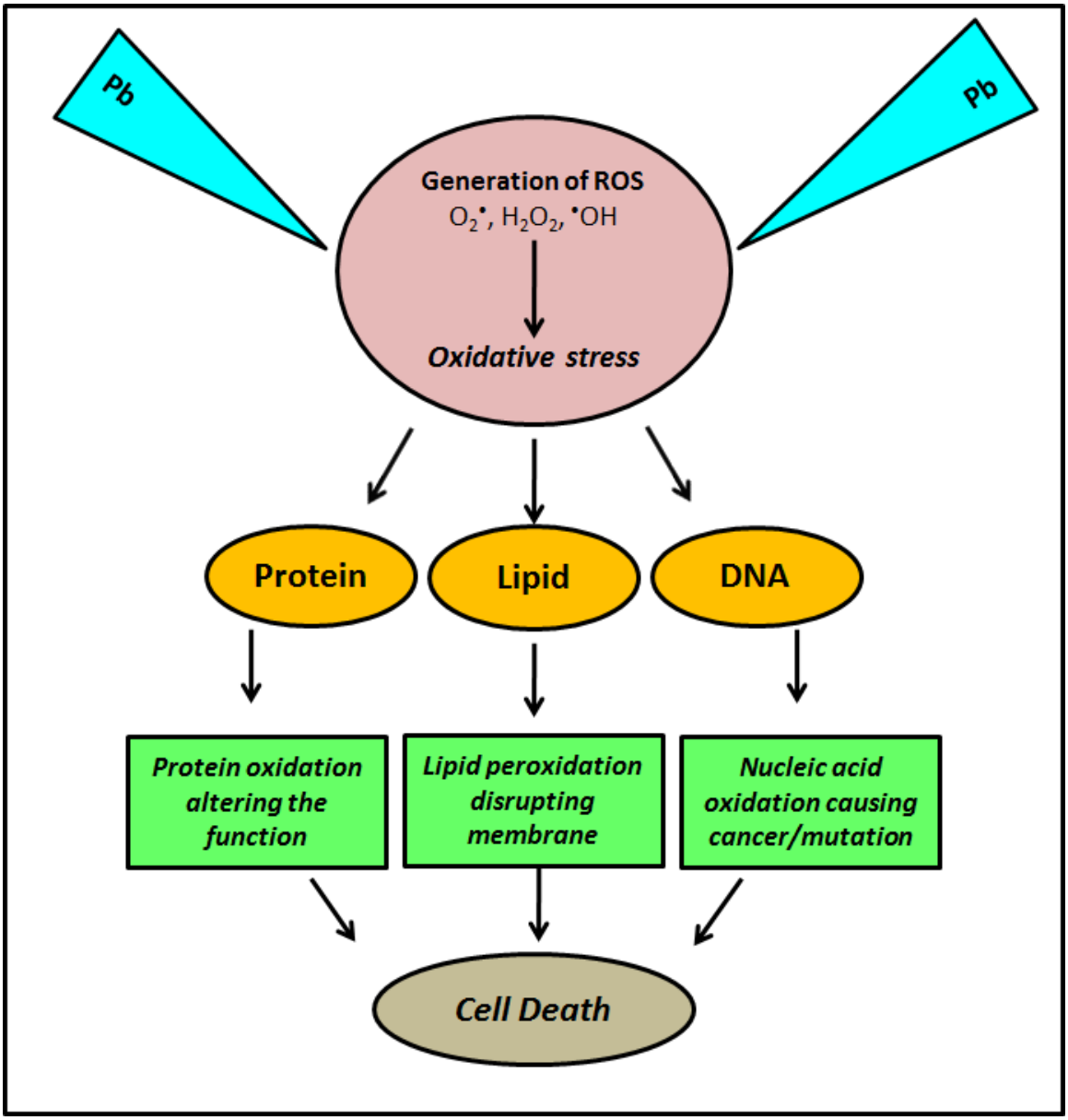

Figure 5.1 Possible mechanism of ROS formation and targets for Pb-induced oxidative stress (after Flora et al., 2012).

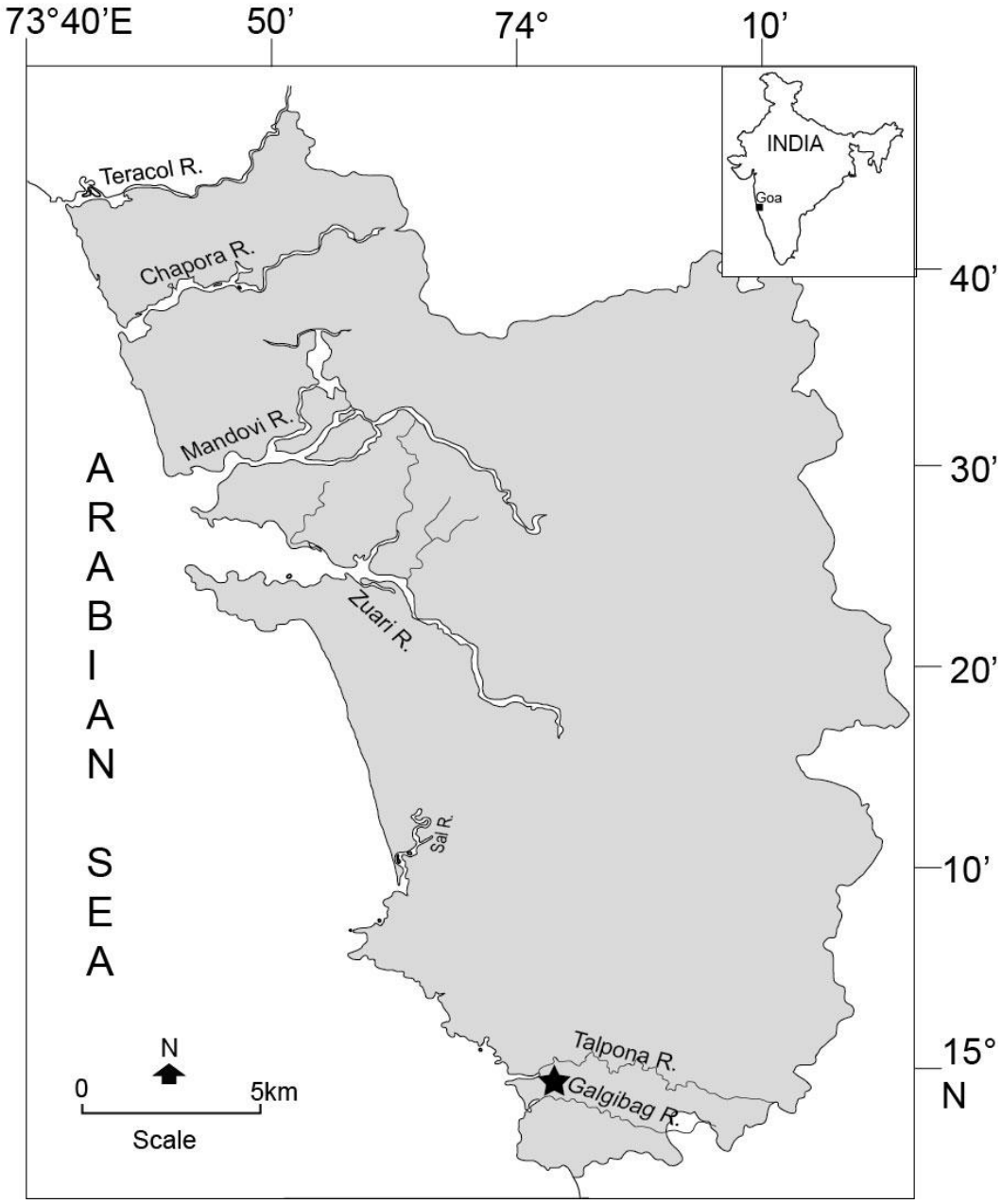

Figure 5.2 The location of sampling site (★) at Galgibag estuary, Goa coast, India.

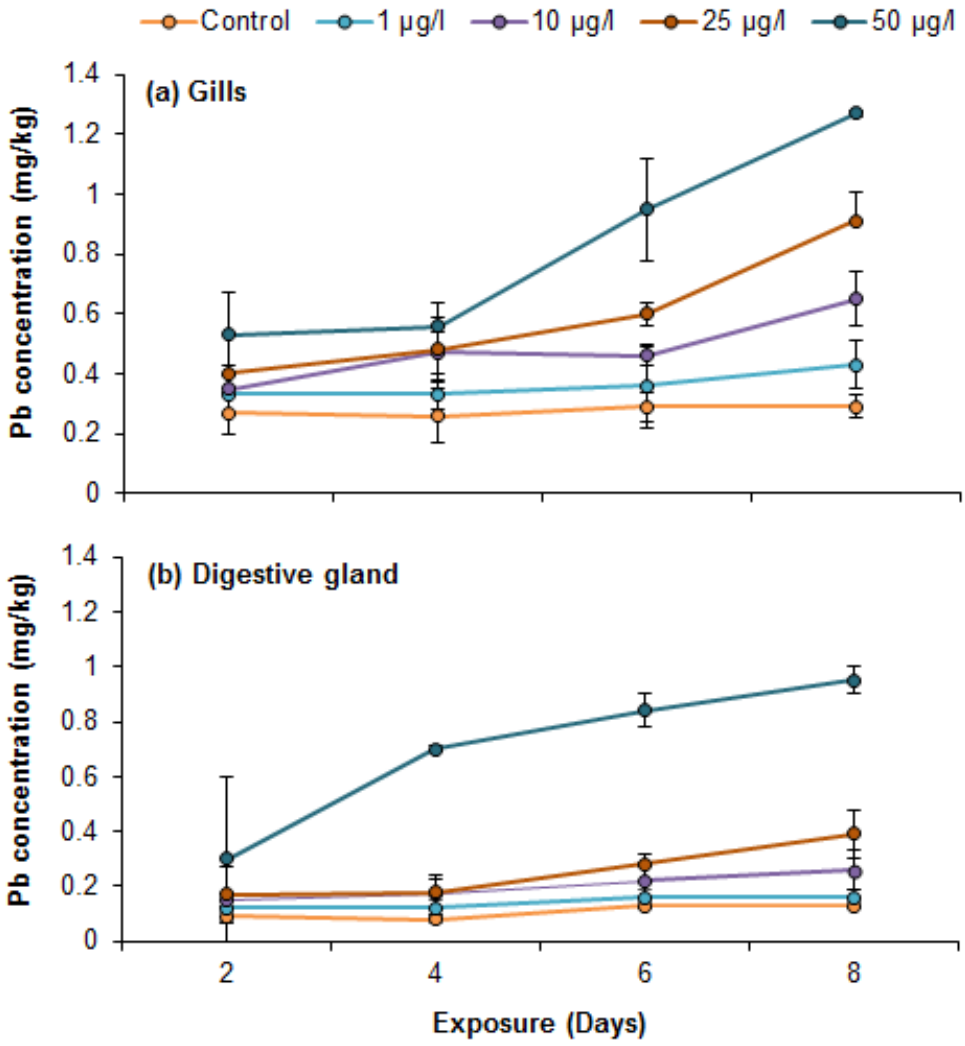

Figure 5.3 Total average Pb concentrations (mg/kg) ± SD (n = 3) in (a) gills and (b) digestive gland of control and exposed *Crassostrea madrasensis* to various concentrations of Pb $(NO_3)_2$ for 8 days.

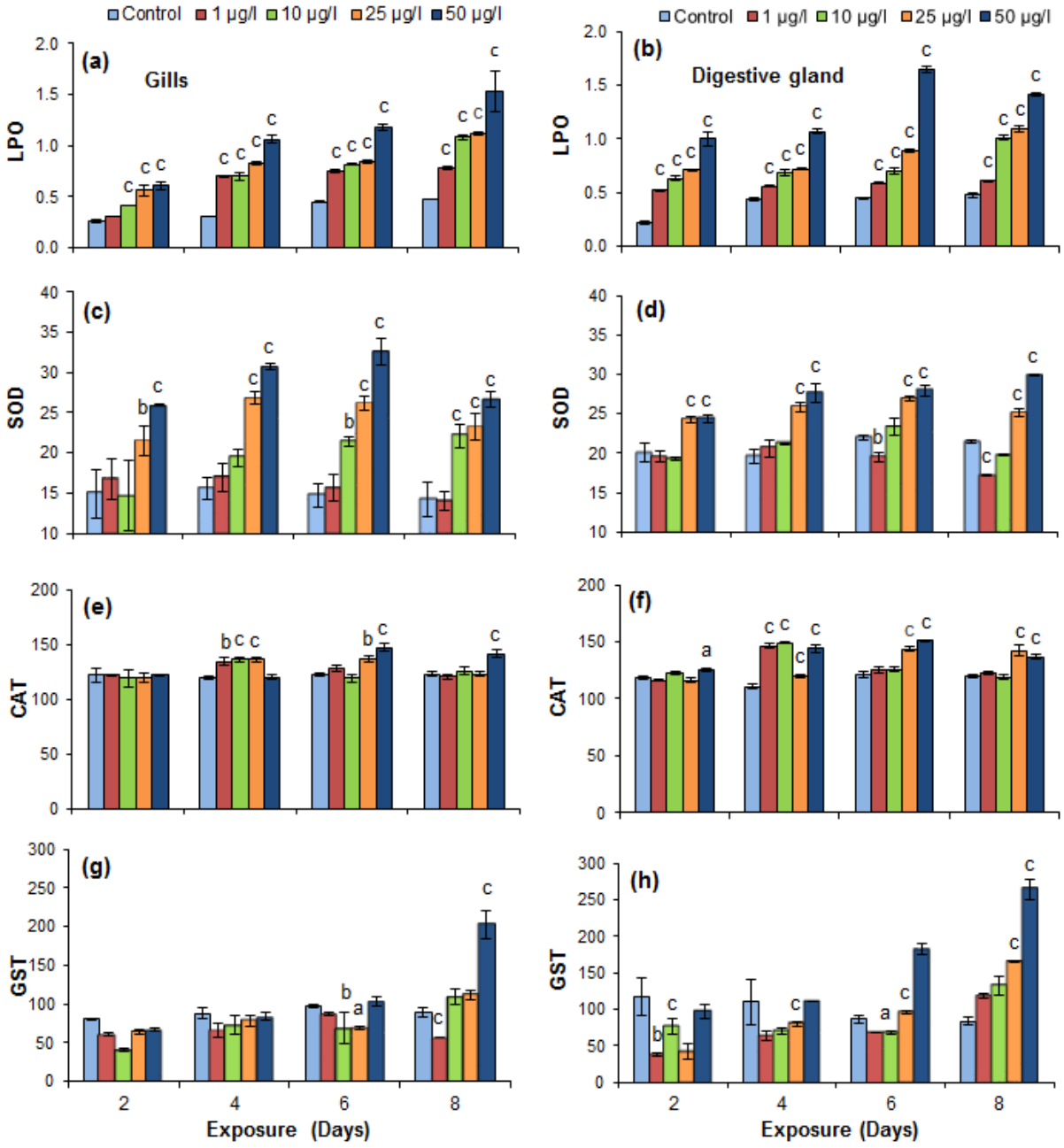

Figure 5.4 Effect of Pb on (a and b) LPO = Lipid peroxidation (nmol TBARS formed/mg protein), (c and d) SOD = Superoxide dismutase (Unit/mg protein), (e and f) CAT = catalase (nkat/mg protein), (g and h) GST = Glutathione-s-transferase (nmol CDNB conjugate formed/min/mg protein) in gills and digestive gland of *Crassostrea madrasensis*. Data are expressed as average ± SD (n = 3). Significance level at $p<0.05$ = a, $p<0.01$ = b, $p<0.001$ = c.

Plate 5.1 Location of sampling site at Galgibag estuary, Goa coast, India.

Plate 5.2 Experimental setup: oyster *Crassostrea madrasensis* exposed to varying Pb concentrations under laboratory condition.

Chapter 6

Summary

Chapter 6

Human Impacts have increased in the coastal areas due to our rapid population growth and substantial development and significant changes in land use in recent time. Consequently, marine ecosystems are being threatened by the discharge of untreated sewage, wastes and industrial effluents which ultimately affect the aquatic life and sustainability of living resources and public health. These wastes carry enormous level of toxicants especially the heavy metals which have the tendency to accumulate into the basic food chain and move up through the higher trophic level thereby threatening marine life and other study region located on the western side of the central west coast of India.

The estuarine and coastal habitat of Goa remain under constant threat from various types of contamination and serve as rich sinks for carbon, nutrients, metals, agricultural waste and other terrigenous matters arising from various anthropogenic activities. Bivalves, particularly oysters (*Crassostrea madrasensis*, *C. gryphoides*, and *Saccostrea cucullata*) are commercially exploited throughout the year from Goa waters. It is therefore, necessary to evaluate the health status of these species to understand their suitability for consumption. In this research, a detailed study on oyster from the Goa coast was carried out with the help of field observation and experimental data.

A survey of oyster beds was conducted along the Goa coast for selection of appropriate sampling sites. Based upon occurrence of major oyster (*C. madrasensis* and *C. gryphoides*) beds which are subjected to different anthropogenic activities, three different sites (i) Chicalim Bay (CB), (ii) Nerul Creek (NC) and (iii) Chapora Bay (ChB) were chosen. Under this investigation, monthly oyster sampling for a period of 14 months

(April 2013–May 2014) was carried out at CB and NC. Whereas at ChB, which was considered as a relatively pristine area (reference site), sampling was restricted once in four months covering three seasons viz. monsoon (July 2013), post-monsoon (November 2013) and pre-monsoon (March 2014). Surface water and sediment samples were collected from the above-mentioned sites for hydrological and geochemical and biological analysis.

Spatial and temporal variations were observed in the physico-chemical parameters of the studied sites. The reason for this variation is influence of southwest monsoon on coastal waters of Goa. Cloud cover, heavy rainfall as well as strong wind during monsoon decrease the summer high temperature and increase relative humidity. Freshwater runoff during rainy season (June – September) reduces water temperature, salinity and pH, and enriches the habitat with dissolved oxygen (DO), nutrients, total suspended solids (TSS), particulate organic carbon (POC). An elevated level of ecotoxicological hazard at CB and moderate toxicological risks at NC were observed. In contrast, ChB was considerably pristine compared to other two sampling sites. Based on the results of physico-chemical parameters, this study conclude that CB was most affected by anthropogenic activities while ChB was appeared to be least affected site.

Apart from physico-chemical parameters, analysis of metal concentrations in tissues of oysters, *C. madrasensis* and *C. gryphoides*, surface sediment, SPM, and surface seawater was carried out. In this study, it was observed that metal values recorded in all the

components at ChB was similar in range with CB and NC. This suggests that the reference site was also impacted due to the fishery activities.

In the present study, surface sediment at all sites depicted significant enrichment of Cu and Ni exceeding the Effect Range Low (ERL) level. The higher concentrations of Cu and Ni in sediments and SPM from all the study sites are indicative of severe contamination of estuarine and associated habitats. Moreover, particulate Ni (at all the sites), Cu (at NC and ChB), Pb (at NC) and Cd (at CB and NC) concentrations were recorded more than its total loadings in surface sediment. This is due to dominance of pebbles, boulders and sand (>79 % sand) at studied sites which hold less metal binding and adsorption ligands such as organic matter in sediment and available more in suspension phase in overlying water column. Further, the concentration of particulate and sediment metals was recorded to be high in monsoon and low in pre-monsoon season at all the sites. This is attributed to the entrance of large amount of metal bound organic and suspended particulate rich land runoff from adjoining areas into the estuarine/marine water bodies during rainy season.

Tissues of both the oyster species showed enrichment of Cu, Cd and to some extent Pb, which was higher than the recommended limits set by international authorities. However, Ni showed less accumulation in tissue. The lower concentration of Ni indicates that Ni does not have strong binding affinity in oyster tissues and thus it is not toxic for the species. While, Cu and Cd values in oyster tissues were several folds higher than its concentration in the ambience. This could be attributed to the metal bioaccumulation

kinetics in oysters, which concentrate metal from surroundings for long period. Further, it exhibited that metals associated with the particulate matter in water column is the main source of metal accumulation in oyster. The present study showed that the levels of metal were higher during the monsoon season in oysters and their ambient environment. Hence, the consumption of oysters needs to be considered carefully with respect to the health hazards posed by the elevated levels of metal contaminants in certain seasons. Therefore, it is suggested that concentration of metal pollutants in coastal and estuarine water bodies to be monitored regularly through observation to ensure the acceptable limits.

To evaluate the toxic effects of Pb, activity of antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), and glutathione-s-transferase (GST) and oxidative damage parameter lipid peroxidation (LPO) were measured in the gills and digestive glands of oysters by exposing them to 1–50 µg/l of Pb $(NO_3)_2$ over a period of 8 days. LPO index increased progressively with increase in Pb concentration (1, 10, 25 and 50 µg/l) in both tissues, gills and digestive gland. Although CAT and SOD activities induced together in the initial phase (up to 6th day), their activities decreased at a later stage of the experiment. In contrast, GST activity increased on 8th day in both the tissues at concentration 10, 25 and 50 µg/l which suggests the compensatory defense mechanism against oxidative stress. The induced antioxidant responses recorded at 25 and 50 µg/l of Pb concentrations suggest the presence of Pb-induced oxidative stress at these higher concentrations. This study concludes that high level of dissolved Pb concentration (>10 µg/l) in surrounding seawater to be harmful to the physiology of the *C. madrasensis*.

The results of present study can help in evaluations of similar ecosystems both nationally and regionally and initiate mitigation measures to reduce metal contaminations in aquatic water bodies. Nevertheless, detailed studies are required to assess the additive effects of these individual metals on commercially important bivalves. Various nondestructive methods and regular monitoring need to be implemented to reduce metal contamination. Bivalve consumers and concerned people in the fisheries industry should be made aware about the metal pollution health hazards associated with fishery products from heavily contaminated habitats. The findings of this research work warrant immediate measures to mitigate the contaminant levels in estuaries and their associated habitats. Management of natural oyster beds is important to insure the contaminant free long-term sustainable yield. Shell planting and regulated harvesting practices, based on sound biological data, would enable the oyster resources to continue contributing to the well-being of the fishermen community that depend on this resource for part of their livelihood.

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Publications

Chicalim bay's marine biodiversity at risk: Experts

NIO Study Points To Magnified Contamination Of Water Body

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Panaji: The Chicalim bay, with its marine biodiversity—specially its oysters—has been more affected by the ecotoxicological impacts of industrial and other human activities along the river as compared to Nerul creek, while the Chapora bay is still pristine, a study by the National Institute of Oceanography (NIO) revealed.

The study, titled 'Evaluation of surface water and sediment quality in Chicalim bay, Nerul creek and Chapora bay', conducted by PS Shenai-Tirodkar, MU Gauns, and Dr ZA Ansari, sampled the surface water and sediments of the three sites.

Results indicated a marked dominance of nutrients, phaeopigments, particulate organic carbon and total suspended solids at the Chicalim bay and the Nerul creek. With its clayey soil, the Chicalim bay has the capacity to absorb metals from the water column. This phenomenon is not prevalent in the Nerul creek and Chapora bay owing to their sandy soil, the report stated.

LIFE UNDER WATER

- Aquatic organisms need oxygen to break down organic matter
- Lead content in water column hinders this process, making the aquatic environment toxic
- Activities of barge construction near oyster beds result in metals dissolving in the water column
- Paints used onboard barges also contain cadmium or lead
- Chicalim bay is more exposed to these compared to Nerul creek and Chapora bay

"With mining-related activities being prominent in Chicalim and its soil's potential to retain the metals, the contamination in the bay is magnified," senior scientist, Dr Mangesh Gauns told **TOI**.

Top of the MIND

He explained that fine sediment particles that remain suspended in the water column, are filtered by grazers in the aquatic system. In the process of filtering, some matter gets accumulated in the grazers, making them poisonous for consumption.

Chicalim bay, being a grazer belt, is therefore most vulnerable.

"The Chicalim bay is highly impacted by anthropogenic activities like barge-building and ore transportation. This poses a risk to its rich marine biodiversity. Consuming these grazers is a health hazard for humans," he added.

The commonly found grazers here are oysters, clams and green mussels.

"Post-monsoon, oysters are harvested in Chicalim, which locals subsequently consume on a daily basis. This can have hazardous effects on their health and could also cause neurological problems," said PhD student, Prachi Shenai-Tirodkar.

Researchers further added that other aquatic life could also be affected by the contaminated water if the bay's environment became toxic. This could, in turn, even impact the fisheries sector.

The different physio-chemical parameters measured in the study also revealed that increasing anthropogenic activities along the banks of River Mandovi and River Zuari introduce domestic and anthropogenic metals and cause contamination in their estuarine regions.

The study suggested monitoring heavy metal concentrations in water, sediments and associated biota, specially oysters, to create a comprehensive pollution database.

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Evaluation of surface water and sediment quality in Chicalim Bay, Nerul Creek, and Chapora Bay from Goa coast, India—a statistical approach

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Abstract To better understand the spatial and temporal variation in surface water and sediment quality, parameters were evaluated from the three sites Chicalim Bay (CB), Nerul Creek (NC), and Chapora Bay (ChB) along the Goa coast, which has major oyster beds. Multivariate analysis such as cluster analysis (CA), Box–Whisker plot (Box plot), and principle component analysis (PCA) revealed that nitrate (NO_3–N), nitrite (NO_2–N), phosphate (PO_4–P), particulate organic carbon (POC), total suspended solids (TSS), dissolved oxygen (DO), and phaeopigments are the responsible parameters for spatio-temporal variability among the studied sites. Results showed an elevated level of ecotoxicological hazard at CB while moderate toxicological risks were observed for organisms at NC. In contrast, ChB was considerably pristine compared with other two sampling sites. Results of present study showed marked dominance of nutrients, phaeopigments, POC, and TSS at CB and NC. The increased levels of these parameters are attributed to the anthropogenic activities which may cause potential risk to humans via consumption of oysters. Therefore, we suggest monitoring heavy metal concentrations in tissue of commercially important oyster species, and their ambient environment (water and sediment) from these estuaries is necessary to create a comprehensive pollution database.

Keywords Environment quality · Oyster beds · Anthropogenic activities · Multivariate analysis · Estuaries

Introduction

Estuaries and coastal ecosystems are among the most valuable and yet highly vulnerable habitats in the world (Jickells 1998). These ecosystems are increasingly becoming threatened due to growing population and exploitation pressure in most parts of the world. Estuaries and coastal ecosystems have greater biodiversity than open ocean regions and are one of the most exploited ecosystems in the world (Blaber 2000). The health of coastal systems of India is being deteriorated due to the rapid introduction of pollutants of physical, chemical, and biological origin, resulting from various anthropogenic activities (Qasim and Kureishy 1986). These pollutants act unfavorably to the aquatic organisms and change their biodiversity and alter the characteristics of the ecosystem (Hall and Ellis 1985; House et al. 1993).

Goa is situated along the central west coast of India between 14° 49′ and 15° 42′ N and 73° 38′ and 73° 24′ E. It has ~105 km coastline intended with bays, creeks, promontories, sea cliffs, estuaries, and world-famous beaches. Nine rivers (Terekhol, Chapora, Baga, Mandovi, Zuari, Sal, Saleri, Talpona, and Galgibag) flow across the state which drains into the Arabian

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Sea. Out of these, Mandovi and Zuari rivers are famous as lifeline of Goa because of their economic importance which covers the 69 % of the geographic area of the state. In Goa, estuaries are used for ore transport, fishing, tourism activities, aquaculture, harbor development, and waste disposal. Such anthropogenic activities may alter the physical, biological, and geochemical conditions of the estuarine system to a considerable extent. Coastal areas also get pressurizes by increasing human population for resource utilization which leads to habitat degradation, fragmentation, and destruction (Gray 1997). Moreover, these estuaries are classified as a "monsoonal estuaries" (Shetye et al. 2007; Vijith et al. 2009), which receives abundant river discharge only during the monsoon and negligible discharge during the non-monsoon period. Edible bivalves, particularly *Crassostrea gryphoides*, *Crassostrea madrasensis*, *Saccostrea cucullata*, *Paphia malabarica*, *Meretrix casta*, and *Villorita cyprynoides* are exploited round the year in Goa. They are confined to the shallow and intertidal regions of estuaries in close vicinity to or in regions influenced by different anthropogenic activity. Population densities of oyster *Crassostrea* spp. were found along the Goa coast in the range of 100–630 individuals/m^2 (Jagtap et al. 2011). Their beds were most predominant in Chapora and Mandovi estuaries in North Goa and Zuari and Talpan estuaries in South Goa. Therefore, it is essential to regularly monitor the ambient environment (surface water and sediment) of oyster species.

To understand the estuarine health status, some measures of reference physico-chemical parameters is often necessary. Due to complexity of relationship and interdependence of key environmental factors, it is difficult to draw a clear conclusion directly. However, statistical analysis can extract the underlying information and explain the structure of data in detail. In recent years, multivariate statistical techniques have been successfully applied to evaluate spatio-temporal variations of physico-chemical parameters caused by natural and anthropogenic factors in coastal waters (Simeonov et al. 2004; Singh et al. 2004; Panda et al. 2006; Shirodkar et al. 2009; Wu et al. 2010; Sahu et al. 2013; Jha et al. 2014).

In the present study, we have used this technique to explain the influence of possible sources (natural and anthropogenic) on water and sediment quality of Chicalim Bay, Nerul Creek, and Chapora Bay from the Goa coast.

Materials and methods

Study area

Based upon occurrence of major oyster beds which are subjected to different anthropogenic activities, three different sites were chosen from the Goa coast, India (Fig. 1). The details of selected sites are as follows:

Chicalim Bay (CB; 15° 24′ 3.52″ N, 73° 51′ 14.24″ E) is located on the southern bank towards confluence of Zuari. This site hosts various shipbuilding industries, yards, workshops, and recreational anthropogenic activities. This site is also exposed to iron ores transportations.

Nerul Creek (NC; 15° 30′ 37.70″ N, 73° 46′ 48.75″ E) opens into the Aguada Bay of Mandovi estuary and extends inside the land in U-shape up to a length of about ~8.5 km. This site is influenced by restaurants discharge, fishing, and other tourism activities and is also exposed to iron ore transportations from mines located upstream.

Chapora Bay (ChB; 15° 36′ 30.43″ N, 73° 44′ 7.19″ E) site is located far from the main city. This site is exposed to major fish landing jetty, sewage disposal from land inhabitant along the bank of river.

Sample collection and analysis

Monthly sampling for a period of 14 months (April 2013–May 2014) was carried out at CB and NC. However, at ChB which was considered a relatively pristine area, samplings were done once in 4 months covering three seasons viz. monsoon (July 2013), post-monsoon (November 2013), and pre-monsoon (March 2014). Surface water and sediment samples were collected from the above-mentioned sites and brought to the laboratory in an ice box for hydrological and geochemical parameter analysis. All the samples were analyzed in duplicates.

Climatological data

The monthly average data of atmospheric temperature, humidity, and rainfall for study period were obtained from the Autonomous Weather Station (AWS) installed at the National Institute of Oceanography (NIO), Dona Paula, Goa.

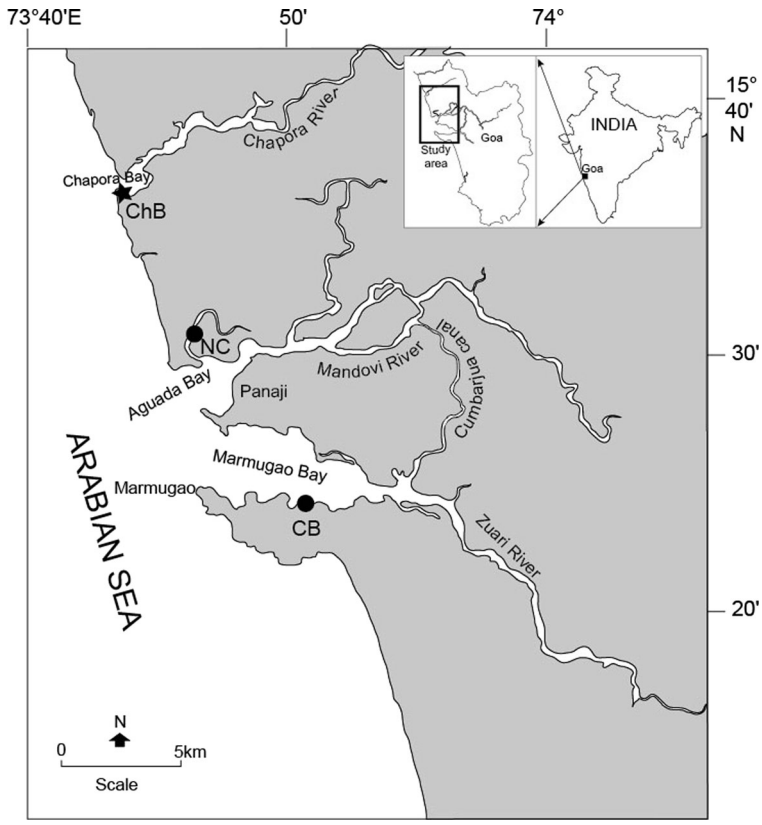

Fig. 1 Map showing sampling sites (*CB* Chicalim Bay in Zuari estuary, *NC* Nerul Creek in Mandovi estuary, *ChB* Chapora Bay in Chapora estuary) along the Goa coast (*circles*, monthly sampling; *star*, seasonal sampling)

Water analysis

Temperature and pH were measured on site using calibrated thermometer and a portable pH meter, respectively. Salinity was determined by a refractometer (ATAGO, S/Milli-E). The samples for dissolved oxygen (DO) were fixed on site and later analyzed according to Winkler's procedure (Parsons et al. 1984). For estimation of chlorophyll *a* (Chl *a*) and phaeopigment, 500 ml of surface water was filtered through the Whatman GF/F filter paper. The pigments were extracted in 90 % acetone (10 ml) for 24 h at 4 °C. Fluorescence was measured before and after acidification with two drops of 1.2 N HCl following the JGOFS protocol (UNESCO 1994). Nutrients such as nitrate (NO_3–N), nitrite (NO_2–N), and phosphate (PO_4–P) and particulate organic carbon (POC) were analyzed by following Parsons et al.'s (1984) method. Total suspended solids (TSS) in water samples (500 ml) were filtered through a pre-weighed 0.45 μm Millipore membrane filter paper. The residue on the filters was dried at 60 °C till constant weight was obtained. The difference between initial and final weight of residue was recorded. Results of TSS (dry weight) were expressed in milligrams per liter as described by Reddy et al. (1994).

Sediment analysis

For measurement of total organic carbon (TOC), sediment samples were decalcified using 1 N HCl, washed thoroughly with deionized water, and dried at 60 °C. The total carbon (TC) and total nitrogen (TN) were analyzed without acid treatment. Following that concentration of TOC, TC and TN were measured using an elemental analyzer (Flash EA 1112 series, Thermo Fisher Scientific) and values were expressed in percentage. For texture analysis, sediment samples were desalinated by washing repeatedly with deionized water and dried at 45 °C. Dried sediment samples (15 g) were first disaggregated using 10 % hexametaphosphate and then treated with 30 % H_2O_2 for removal of organic

matter. The sample was put through a 63 μm sieve (USA standard testing sieve, A.S.T.M.) to separate the sand fraction from the bulk sediment and was oven dried at constant temperature 60 °C and weighed to calculate sand fraction percentage. The remaining mud (silt + clay) fraction from the same was analyzed by laser size particle analyzer (Malvern Mastersizer 2000). The data are presented as weight percentage (wt.%) in this study.

Statistical analysis

The basic statistics were applied on raw data set. The mean ± standard deviation (SD), maximum value, and minimum value of surface water and sediment parameters were taken separately for each site using Microsoft Office Excel 2007. Pearson's correlation test was used to assess the significance of association between environmental parameters at each site. To determine the significance of spatial and temporal variation in water and sediment parameters, two-way analysis of variance (ANOVA) was used. Further statistical analysis applied only on surface water parameters as the sediment parameters did not show much significant variation in the two-way ANOVA analysis. Hereafter, values of surface water parameters at CB and NC were taken as an average of 4 months in each seasons: monsoon (Mon: June–September), post-monsoon (PostM: October–January), pre-monsoon2 (PreM2: February–May 2014) except pre-monsoon1 (PreM1: April–May 2013). To minimize the effects of differences in measurement units and to deliver the data dimensionless, all the water variable values were normalized. Cluster analysis (CA) and principle component analysis (PCA) were performed on normalized data. Based on CA, raw data were examined using box and whisker plots. All statistical computations were made using STATISTICA 8 (StatSoft, Inc., USA) and PRIMER 6 (Primer-E Ltd., Plymouth, UK) software.

Results and discussion

The climate of Goa is generally tropical with humid conditions. During the study period, the atmospheric temperature ranged from 25.5 to 29 °C. The average humidity of ambient atmosphere reached up to 77 %. The total rainfall received during the study period at Goa coast was about 7501 mm (Fig. 2).

Environmental conditions such as temperature, pH, salinity, food availability, nutrient levels, suspended load, etc. have considerable impact on the physiology of sedentary, filter feeder oyster species (Lenihan 1999). Therefore, in the present study, attempt has been made to decide the suitability of a site for oyster consumption from these regions by measuring the environmental variables. The annual mean ± SD with minimum and maximum values recorded for each of the measured variables from sampling sites are shown in Table 1. Under hydrological observations, the surface water temperature observed within the normal tropical ocean ranged between 28 and 29.4 °C. However, lowest values of pH and salinity were observed during monsoon compared with non-monsoon period at all the three sites. Increased rainfall and dilution of seawater by the freshwater influx led to decreased salinity (1–2 psu) in surface water. During monsoon, heavy land runoff contributes large amount of organic material-forming organic acids due to decomposition, which may be one of the reasons responsible to a decrease in pH at all the three sites. James and Najmuddin (1986) also observed similar phenomenon in Palk Bay, Mandapam. During monthly observations, relatively higher concentrations of DO, Chl *a*, and phaeopigments were observed at CB than NC. Dissolved oxygen showed biologically stressful levels (hypoxic threshold) in some pre-monsoon months with minimum values of 2.54 and 2.97 mg/l at CB and NC, respectively. Further, NO_3–N, NO_2–N, PO_4–P, POC, and TSS were found higher at site CB. In sediment analysis, mean value of sand percentage (98.3 %) was found to be higher whereas TOC (0.11 %) and TC (0.18 %) were very low at ChB among the three sites. This low carbon content could be attributed to the dominant sandy nature of the sediment. Comparatively higher average value of TOC (1.36 %) and TC (1.59 %) of sediment were observed at NC, though percentage of clay is less compared with CB site. This may be attributed to mangrove vegetation at NC which contributed more input of organic material from the catchment area into sediment of the bay. Concentrations of TN were also low in surface sediment at CB (0.01 %) and NC (0.03 %) whereas at ChB, it was below detection limit. Pradhan et al. (2014) also reported low values of TC and TN from Chapora estuary in comparison with Mandovi and Zuari estuaries. Shynu et al. (2015) found similar results of OC (0.24–1.34 %)

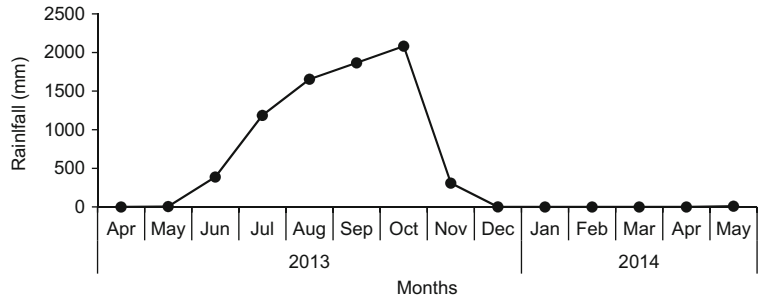

Fig. 2 Monthly variations in rainfall recorded in Goa coast during 2013–2014

and TN (0.01–0.07 %) from Mandovi and Zuari estuaries.

Pearson correlation matrix at the significant level of $P < 0.05$ was applied to uncover the relationship among the ecological status indicators (Tables 2, 3, and 4). At all the three study sites, the values of nutrients showed significant negative relationship with temperature, salinity, and pH. The reason for this relationship could be the terrestrial runoff containing domestic waste in the waters of study sites. In contrast, at CB, a significant positive relationship was observed among POC, chl *a*, and phaeopigments (Table 2), suggesting occurrence of productive process resulting in enhancement of the oyster population in this bay. However, this correlation increases the potential health hazard to human as oyster in this bay could assimilate more pollutant (metals, bacteria, etc.) along with food particles. The observed values of TSS showed a significant positive correlation with NO_3–N, PO_4–P, DO, and phaeopigments; whereas, a negative relationship was observed with salinity. This

Table 1 Physico-chemical parameters (mean ± SD, min., max.) at all the sites during study period 2013–2014

Parameters	CB				NC				ChB			
	Mean	SD	Min.	Max.	Mean	SD	Min.	Max.	Mean	SD	Min.	Max.
Water parameters												
Temperature (°C)	29.42	2.58	25.00	34.00	28.86	1.73	26.00	32.00	28.00	1.80	26.00	29.50
pH	7.90	0.42	6.75	8.60	7.74	0.60	6.23	8.70	7.45	1.04	6.25	8.10
Salinity (psu)	24.86	10.62	2.00	34.00	23.26	11.20	1.00	34.00	17.33	13.28	2.00	25.00
DO (mg/l)	5.46	1.21	2.54	7.41	4.87	1.16	2.97	6.58	5.73	0.38	5.36	6.12
Chl *a* (μg/l)	7.34	3.49	1.33	12.34	4.77	2.30	2.02	8.97	3.10	0.80	2.27	3.87
Phaeopigment	9.85	8.85	3.76	34.03	2.43	1.28	0.73	5.82	1.71	1.19	0.63	2.98
NO_3–N (μmol/l)	6.86	9.09	0.94	27.49	3.78	6.48	0.01	24.48	8.39	13.54	0.45	24.02
NO_2–N (μmol/l)	0.36	0.24	0.08	0.97	0.28	0.24	0.10	1.04	0.21	0.16	0.11	0.39
PO_4–P (μmol/l)	2.14	2.61	0.43	9.18	1.14	1.31	0.06	3.96	1.38	1.90	0.21	3.57
POC (μg C/l)	4349.30	2500.17	2061.03	11,325.60	2224.60	1668.17	901.45	6773.98	1512.84	347.81	1137.40	1824.08
TSS (mg/l)	239.68	260.72	30.27	870.53	29.96	20.08	11.13	86.07	17.39	21.64	3.70	42.33
Sediment parameters												
Sand (%)	79.49	12.55	57.31	97.04	80.09	6.31	66.19	90.06	98.34	0.97	97.25	99.11
Silt (%)	18.11	11.05	2.43	37.87	17.60	5.49	8.88	29.73	1.51	0.87	0.80	2.49
Clay (%)	2.34	1.54	0.52	5.35	2.29	0.87	1.06	4.08	0.14	0.09	0.09	0.25
TOC (%)	0.85	0.60	0.16	2.05	1.36	0.51	0.54	2.23	0.11	0.03	0.08	0.13
TC (%)	1.39	0.91	0.17	3.57	1.59	0.66	0.64	3.01	0.18	0.10	0.07	0.28
TN (%)	0.01	0.03	0.00	0.12	0.03	0.07	0.00	0.18	0.00	0.00	0.00	0.00

CB Chicalim Bay, *NC* Nerul Creek, *ChB* Chapora Bay, *SD* standard deviation, *Min.* minimum, *Max.* maximum

Table 2 Correlation coefficient (r values) between physico-chemical parameters at Chicalim Bay during study period

	Temp.	pH	Salinity	DO	Chl *a*	Phaeo.	NO_3–N	NO_2–N	PO_4–P	POC	TSS	Sand	Silt	Clay	TOC	TC	TN
Temp.	1																
pH	0.099	1															
Salinity	0.631*	0.245	1														
DO	−0.322	−0.237	−0.532	1													
Chl *a*	0.325	0.633*	−0.085	0.091	1												
Phaeo.	−0.106	0.309	−0.24	0.215	0.597*	1											
NO_3–N	−0.716**	−0.302	−0.966***	0.535*	−0.012	0.181	1										
NO_2–N	−0.243	0.265	−0.148	0.116	0.334	0.758**	0.226	1									
PO_4–P	−0.467	−0.164	−0.714***	0.255	0.215	0.552*	0.741**	0.563*	1								
POC	−0.368	0.588*	−0.337	0.12	0.567*	0.683**	0.278	0.43	0.271	1							
TSS	−0.479	−0.333	−0.788***	0.588*	0.175	0.633*	0.784***	0.509	0.806***	0.365	1						
Sand	0.057	0.205	−0.385	0.107	0.529	0.301	0.307	−0.07	0.311	0.526	0.279	1					
Silt	−0.06	−0.217	0.382	−0.126	−0.536*	−0.3	−0.302	0.078	−0.297	−0.525	−0.272	−0.999***	1				
Clay	−0.045	−0.127	0.375	−0.005	−0.435	−0.283	−0.325	0.006	−0.387	−0.478	−0.293	−0.966***	0.955***	1			
TOC	−0.057	−0.249	0.344	−0.167	−0.485	−0.328	−0.233	0.064	−0.191	−0.496	−0.24	−0.874***	0.887***	0.767***	1		
TC	−0.052	−0.457	0.253	−0.201	−0.525	−0.342	−0.132	−0.039	−0.073	−0.53	−0.133	−0.729**	0.748**	0.586*	0.929***	1	
TN	−0.047	0.001	0.139	0.004	0.001	−0.142	−0.186	−0.271	−0.178	−0.215	−0.198	−0.405	0.383	0.561*	0.260	0.186	1

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$—levels of significance

Table 3 Correlation coefficient (r values) between physico-chemical parameters at Nerul Creek during study period

	Temp.	pH	Salinity	DO	Chl *a*	Phaeo.	NO_3–N	NO_2–N	PO_4–P	POC	TSS	Sand	Silt	Clay	TOC	TC	TN
Temp.	1																
pH	0.379	1															
Salinity	0.469	0.507	1														
DO	−0.28	0.185	−0.4	1													
Chl *a*	0.09	−0.507	−0.31	−0.509	1												
Phaeo.	0.423	0.061	0.183	−0.445	0.592*	1											
NO_3–N	−0.531	−0.802***	−0.760**	0.196	0.305	−0.159	1										
NO_2–N	−0.583*	−0.575*	−0.503	0.079	0.215	−0.053	0.849***	1									
PO_4–P	−0.608*	−0.735**	−0.738**	−0.01	0.49	−0.081	0.727*	0.505	1								
POC	−0.421	0.118	−0.543*	0.371	−0.151	−0.372	0.182	0.227	0.353	1							
TSS	0.234	−0.121	−0.136	−0.432	0.737**	0.815***	0.058	0.125	0.289	0.092	1						
Sand	−0.103	−0.652*	−0.165	−0.471	0.487	0.218	0.43	0.26	0.291	−0.453	0.155	1					
Silt	0.087	0.635*	0.165	0.447	−0.47	−0.211	−0.418	−0.234	−0.275	0.464	−0.141	−0.998***	1				
Clay	0.181	0.710**	0.135	0.605*	−0.53	−0.233	−0.468	−0.386	−0.364	0.372	−0.215	−0.944***	0.924***	1			
TOC	−0.406	0.214	−0.061	0.286	0.074	−0.103	−0.131	0.039	0.222	0.415	0.113	−0.563*	0.569*	0.515	1		
TC	−0.316	0.489	0.133	0.376	−0.229	−0.199	−0.357	−0.135	−0.048	0.437	−0.06	−0.720**	0.721**	0.687**	0.936***	1	
TN	−0.303	0.307	0.089	0.013	−0.021	0.279	−0.203	0.150	−0.009	0.441	0.431	−0.300	0.313	0.210	0.543*	0.587*	1.000

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$—levels of significance

Table 4 Correlation coefficient (*r* values) between physico-chemical parameters at Chapora Bay during study period

	Temp.	pH	Salinity	DO	Chl *a*	Phaeo.	NO_3–N	NO_2–N	PO_4–P	POC	TSS	Sand	Silt	Clay	TOC	TC
Temp.	1															
pH	0.973	1														
Salinity	0.961	0.999*	1													
DO	−0.982	−0.912	−0.891	1												
Chl *a*	0.728	0.867	0.89	−0.586	1											
Phaeo.	0.588	0.759	0.789	−0.425	0.983	1										
NO_3–N	−0.958	−0.998*	−1.000**	0.887	−0.894	−0.795	1									
NO_2–N	−0.948	−0.996	−0.999*	0.871	−0.909	−0.815	0.999*	1								
PO_4–P	−0.948	−0.996	−0.999*	0.871	−0.909	−0.815	0.999*	1.000***	1							
POC	−0.92	−0.804	−0.775	0.977	−0.401	−0.224	0.77	0.747	0.747	1						
TSS	−0.975	−1.000**	−0.998*	0.915	−0.863	−0.754	0.998*	0.995	0.995	0.809	1					
Sand	−0.863	−0.722	−0.688	0.943	−0.282	−0.098	0.682	0.657	0.657	0.992	0.728	1				
Silt	0.873	0.736	0.703	−0.949	0.301	0.118	−0.697	−0.672	−0.672	−0.994	−0.742	−1.000**	1			
Clay	0.739	0.563	0.523	−0.853	0.076	−0.111	−0.516	−0.486	−0.486	−0.944	−0.57	−0.978	0.974	1		
TOC	0.994	0.942	0.925	−0.997	0.649	0.496	−0.922	−0.908	−0.908	−0.957	−0.945	−0.913	0.921	0.808	1	
TC	0.978	0.903	0.882	−1.000*	0.569	0.406	−0.878	−0.861	−0.861	−0.982	−0.907	−0.949	0.955	0.863	0.995	1

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$—levels of significance

Table 5 Two-way ANOVA results (*P* values) of physico-chemical parameters

Parameters	Seasonal	Site wise	Site*season
Temperature	*0.000*	0.076	0.594
pH	*0.000*	0.122	*0.001*
Salinity	*0.000*	0.156	0.541
DO	*0.001*	*0.011*	0.718
Chl *a*	0.994	*0.02*	*0.004*
Phaeopigment	0.281	*0.000*	*0.005*
NO_3–N	*0.000*	*0.04*	*0.006*
NO_2–N	*0.000*	0.227	0.229
PO_4–P	*0.025*	0.463	0.907
POC	*0.000*	*0.000*	*0.029*
TSS	*0.000*	*0.000*	*0.000*
Sand	*0.020*	0.368	*0.0001*
Silt	*0.029*	0.356	*0.0001*
Clay	*0.029*	0.592	*0.004*
TOC	0.081	*0.000*	*0.015*
TC	0.336	0.465	0.093
TN	0.929	*0.004*	*0.032*

Level of significance: $P<0.05$

positive correlation indicates that the nutrient and oxygen rich freshwater carries suspended load into the estuary. At NC, POC was negatively correlated with salinity (Table 3). This is attributed to the particulate organic matter which enters the water body along with the freshwater efflux. There is no significant relationship observed between POC and TSS at any of the study sites. Further, the sediment analysis showed a highly significant (P = 0.000) positive correlation between TOC and TC at CB as well as at NC. However, a non-significant relationship was observed at ChB (Table 4) which may be attributed to the sandy nature of sediment.

Two-way ANOVA was used to test the differences in temporal and spatial variation in parameters (Table 5). The values of ANOVA demonstrated significant differences ($P < 0.05$) among the sites, seasons, and a site and season interaction. NO_3–N, POC, and TSS showed a significant ($P < 0.05$) seasonal and site-wise variation. However, a non-significant seasonal variation was observed among TOC, TC, and TN of sediment.

Cluster analysis was done using Euclidean distance test to detect the similarity in the water samples from three localities in four different seasons. Five distinct groups (group A: CB Mon; group B: CB PreM1 and NC PreM1; group C: NC PreM2, ChB PostM, ChB PreM, and NC PostM; group D: CB PreM2 and CB PostM; group E: NC Mon and ChB Mon) were formed based on the seasonal variation in the studied sites (Fig. 3). In group A, only CB Mon was present while CB PreM1 and NC PreM1 clustered in another group (group B). Sites CB and NC showed similar characteristics in first pre-monsoon although anthropogenic activities are different at these sites. Group C included NC PreM2, ChB PostM, ChB PreM, and NC PostM. This group showed more similarity between NC and ChB sites in pre- and post-monsoon except during first pre-monsoon (PreM1) at NC. CB PreM2 and CB PostM formed another group (group D). Overall, site CB formed a two separate clusters such as groups A and D in Monsoon and non-monsoon period, respectively. Group E comprised NC Mon and ChB Mon. The data analyzed in the present

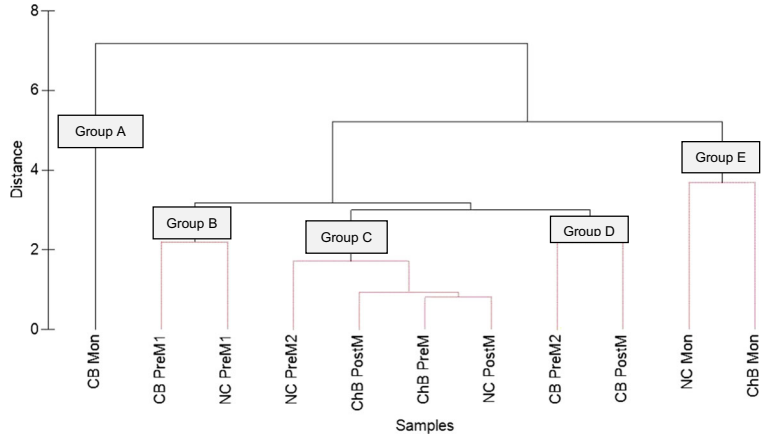

Fig. 3 Dendrogram based on hierarchical clustering method for seasonal variation in water samples at three sites

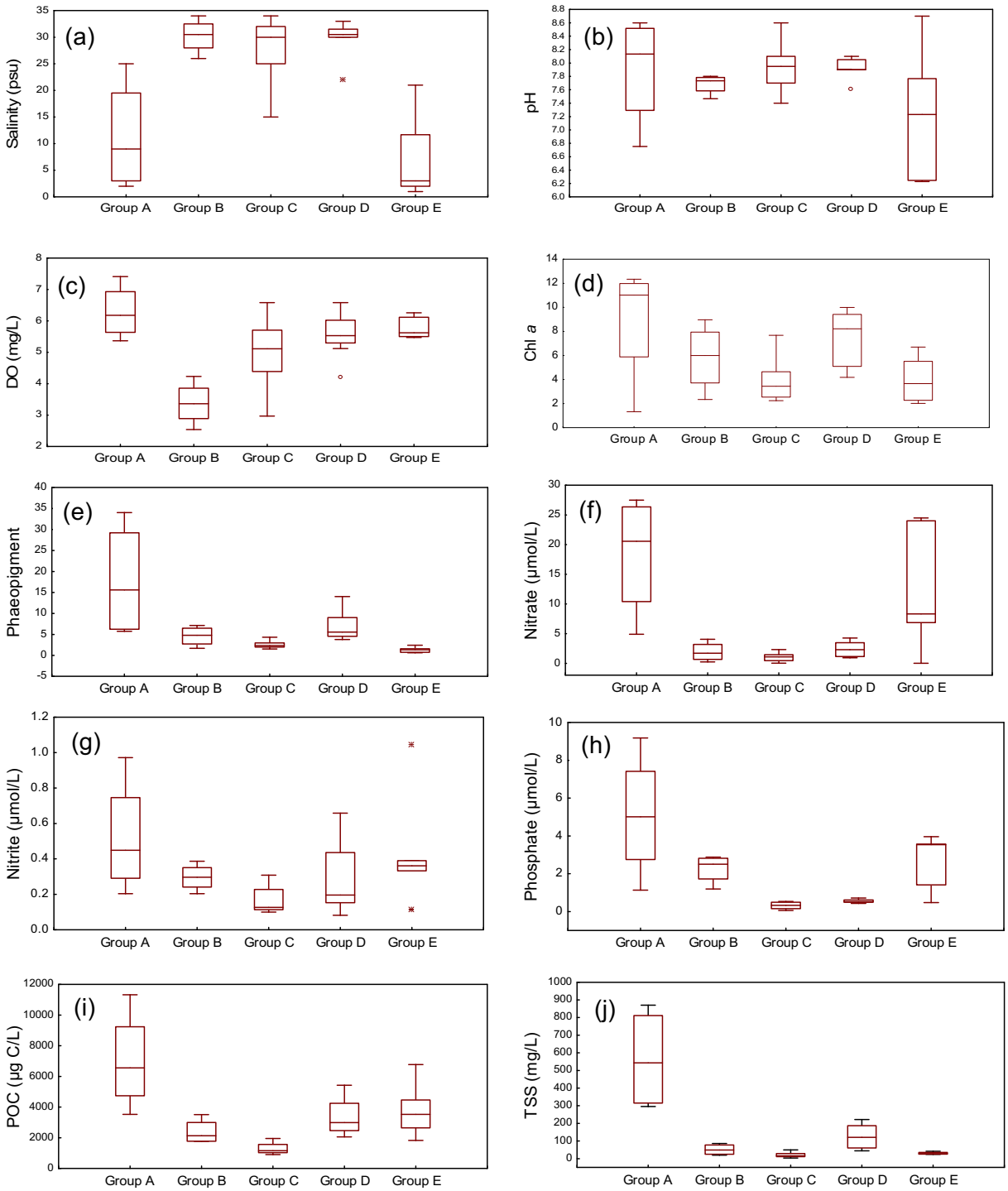

Fig. 4 Box–Whisker plot for **a** salinity, **b** pH, **c** DO, **d** Chl *a*, **e** phaeopigment, **f** nitrate, **g** nitrite, **h** phosphate, **i** POC, and **j** TSS in five groups

study clearly shows that the monsoon clusters are widely separated from non-monsoon. The reason for the observed disparity between the two seasons (monsoon and non-monsoon) could be due to the

influence of south-west monsoon on the hydrology of studied water bodies.

Variables responsible for group formation in CA are explained by Box-Whisker plots (Fig. 4). From the plots, it is observed that most of the parameters are showing wide ranges of variation in group A followed by group E. The reason for the observed variations could be explained by higher influence of land runoff in monsoon period in the above-mentioned groups. On other hand, variability in the concentrations of NO_3–N, NO_2–N, PO_4–P, POC, and TSS are less in group C, which suggests that in non-monsoon season, NC and ChB sitse do not show much difference in environmental conditions. Presence of low DO values in PreM1 at CB and NC water bodies was the main responsible factor to differentiate group B from other groups (Fig. 4c). However, group D which is characterized by higher values of phaeopigment, NO_3–N, NO_2–N, POC, and TSS indicates poor water quality at CB in pre- and post-monsoon seasons. The characteristic values observed at CB could be due to commercial and industrial activities such as fishing, ship building activities, and iron ore transportation at this site. These activities enhance turbulence in the water which causes re-suspension of sediment. As a result, this phenomenon increases the phaeopigment levels in water, since the bottom sediments contain higher quantity of degrading pigments (Anand et al. 2014). The geomorphology of CB may be another reason for the observed values at this site since more ingress of sea water occurs during daily tidal circulations at CB (Selvakumar et al. 1980). Mining rejects is also one of the major sources of NO_3 in the estuaries of Goa (De Souza 1983).

The PCA approach was used to extract the important driving factors that are responsible for spatial and temporal variations in water quality. Results of PCA are presented in Table 6. Based on the eigenvalue (>1), first three principal components (PCs) were retained as they explained 89.8 % of the total variability in the hydrological parameters. Further, comparison between loading and scoring on each PC have helped to identify the relationship between the variables and samples. The PC1 explained 52.1 % of the variance and is associated with all nutrients (NO_3–N, NO_2–N, and PO_4–P), POC, and TSS which showed a negative loading with salinity. The scoring on PC1 showed that all the three sites during monsoon were influenced by high concentrations of these parameters. This indicates that all the studied sites (CB, NC, and ChB) are affected by nutrient-rich freshwater runoff from land and resuspended fine-grained organic-rich sediments during the monsoon period. Similar results were obtained by other researchers when they evaluated the water quality of estuarine regions of Goa (Sardessai and Sundar 2007; Anand et al. 2014). The second PC (PC2) described 28.5 % of the variability and characterized by positive loading of Chl *a*, pH, and phaeopigments. Site CB in all the seasons and site NC during PreM1 showed the

Table 6 PC loadings and scores of water parameters at sampling sites during study period 2013–2014

Variables	PC1	PC2	PC3	Samples	Score1	Score2	Score3
Temperature	0.314	0.291	0.107	CB PreM1	0.812	0.346	−1.750
pH	0.176	0.440	0.246	CB PreM2	1.150	1.540	0.529
Salinity	0.331	0.310	−0.206	NC PreM1	0.618	0.401	−2.010
DO	−0.211	−0.068	0.839	NC PreM2	2.120	−0.091	−0.322
Chl *a*	−0.139	0.451	−0.131	ChB PreM	2.020	−0.487	0.866
Phaeopigment	−0.260	0.422	0.023	CB Mon	−5.540	2.500	0.269
NO_3–N	−0.372	−0.227	0.031	NC Mon	−1.660	−1.320	0.178
NO_2–N	−0.359	0.033	−0.201	ChB Mon	−2.760	−4.140	−0.017
PO_4–P	−0.380	−0.038	−0.340	CB PostM	−0.026	1.750	0.561
POC	−0.337	0.281	0.030	NC PostM	1.680	−0.088	0.628
SS	−0.323	0.326	0.071	ChB PostM	1.580	−0.423	1.060
Eigenvalues	5.73	3.13	1.02				
% variation	52.1	28.5	9.2				
Cumulative %	52.1	80.6	89.8				

highest values of Chl *a*, pH, and phaeopigments. The third PC (PC3) accounting for 9.2 % of the variance is mainly due to positive loading of DO. High loading of DO was observed at all the three sites during PostM period. Additionally, NC and ChB showed strong relationship with DO on PC3 in PreM2 and in PreM, respectively. Variables Chl *a*, pH, and DO showed positive correlation on PC2 and PC3, respectively, mostly in pre- and post-monsoon seasons. Generally, these variables are involved in various biological processes, such as photosynthesis, respiration activities and organic matter decomposition, etc. (Zang et al. 2011) which also influence the water quality. Similar results were observed in waters samples at CB throughout the year. From the PCA results, it is evident that in all the seasons, site CB is more influenced by land-based anthropogenic activities than other two sites (NC and ChB).

Conclusion

The different physico-chemical parameters measured in present study have been used as an indicator for potential pollution in all the three study sites (CB, NC, and ChB). Based on the results of the present study, we can conclude that CB is most affected by anthropogenic activities while ChB is considerably pristine compared with the other two sampling sites. Further, increasing anthropogenic activities along the banks of Mandovi and Zuari rivers introduce domestic and anthropogenic metals and cause the contamination in their estuarine regions (Pradhan and Shirodkar 2011). Previous studies had reported the metal contamination in Mandovi (Algarsamy 2006; Shynu et al. 2013; Veerasingam et al. 2015) and Zuari (Kessarkar et al. 2013) estuaries due to mining and other anthropogenic activities which occurs along the bank of rivers throughout the year. This attracts the urgent need to monitor more parameters such as heavy metal concentrations in water, sediment, and associated biota especially commercially important oyster species from these areas to create a comprehensive pollution database.

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Concentrations of Heavy Metals in Commercially Important Oysters from Goa, Central-West Coast of India

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Abstract The major beds of oyster along the central-west coast of India are exposed to different anthropogenic activities and are severely exploited for human consumption. In this viewpoint, tissues of oyster *Crassostrea madrasensis, C. gryphoides* and *Saccostrea cucullata* were analyzed for Cu, Ni, Cd and Pb concentrations (dry weight) from Chicalim Bay, Nerul Creek and Chapora Bay in pre-monsoon, monsoon and post-monsoon seasons. A higher concentration of Cu (134.4–2167.9 mg kg^{-1}) and Cd (7.1–88.5 mg kg^{-1}) was found, which is greater than the recommended limits in all the three species (and sites). Moreover, significant ($p < 0.05$) variations were observed for all the metals concentrations among the species, seasons and sites. The high concentrations of Cd and Cu in tissues of edible oyster pose a threat to human health. Therefore, continuous monitoring, people awareness and a stringent government policy should be implemented to mitigate the metal pollution along the studied sites.

Keywords Metal pollution · Estuaries · Bioaccumulation · Oysters · Seafood

Metal pollution in aquatic ecosystems, due to increasing levels of contaminants, represents a serious and growing problem worldwide. Metals, especially heavy metals, are serious pollutants due to their toxicity (at high concentration), persistence and bioaccumulation in aquatic organisms (Rainbow 2002). Marine bivalves such as oysters, mussels and clams have been widely used in biomonitoring programs. For example "Mussel Watch" programs use bivalves due to their ability to accumulate and tolerate high concentrations of heavy metals compared to other marine organisms (UNEP 1993). Among the bivalves, oysters (filter feeder) are well known as a universal sentinel accumulator of both essential and non-essential elements from the ambient environment (Amiard et al. 2008). Consequently, oysters from contaminated sites serve a potential risk to human health. Nevertheless, worldwide, oysters are a highly esteemed nutritious seafood, as they constitute rich source of proteins and a number of essential elements (Asha et al. 2014).

India is fortunate to have large resources of oysters. In 2008, 2400 tonnes of oyster production (http://www.fao.org/fishery/countrysector/naso_india/en) was recorded from the Indian coast. *Crassostrea* and *Saccostrea* are the major species occurring along the coastal waters of India (Asha et al. 2014). Edible oysters such as *C. madrasensis* (Preston 1916), *C. gryphoides* (Schlotheim 1813), and *S. cucullata* (Born 1778) are under severe exploitation along the Goa coast. Since estuaries of Goa receives numerous deposits of heavy metals from its Fe-Mn ores industries, barge building activities and waste disposals from the adjacent human settlements, high concentrations of heavy metals have been reported from marine components (sediment, suspended particulate matter and seawater) of Goa (Alagarsamy 2006; Kessarkar et al. 2013; Veerasingam et al. 2015; Prajith et al. 2016).

Although heavy metals concentrations in waters of Goa is known, no such study has been conducted on oyster species. Therefore, it is necessary to carry out a study concerning heavy metals levels in commercially important oyster species that are harvested regularly for human consumption. Keeping this aspect in forefront the present study has been undertaken with the following objectives (1) to determine

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levels of essential metals: copper (Cu), nickel (Ni) and non-essential metals: cadmium (Cd) and lead (Pb) in the tissue of three oyster species (*C. madrasensis, C. gryphoides*, and S. *cucullata*) and (2) to find out whether the oysters occurring along the coastal waters of Goa are safe for human consumption.

Materials and Methods

Three sites including a reference site (where oysters are harvested on regular basis for human consumption) were chosen from the Goa coast, India (Fig. 1) to determine the heavy metals concentrations in oyster species. The details of selected sites are as follows:

Chicalim Bay (CB) (15°24′3.52″ N, 73°51′14.24″ E) is located on the southern bank towards confluence of Zuari. This site hosts various ship and barge building industries, yards, workshops, anthropogenic activities and iron ores transportations. Nerul Creek (NC) (15°30′37.70″ N, 73°46′48.75″E) opens into the Aguada Bay of Mandovi Estuary, extends inside the land in U-shape up to a length of about ~8.5 km. This site is under the influence of fishing and other tourism activities. Also, this creek opens into the Mandovi River which is used for iron ores transportation from mines located upstream. Chapora Bay (ChB) (15°36′30.43″N, 73°44′7.19″E) a reference site located far from the main city. Unlike the other two sites, this site is not influenced by mining activity and has no ship building activities.

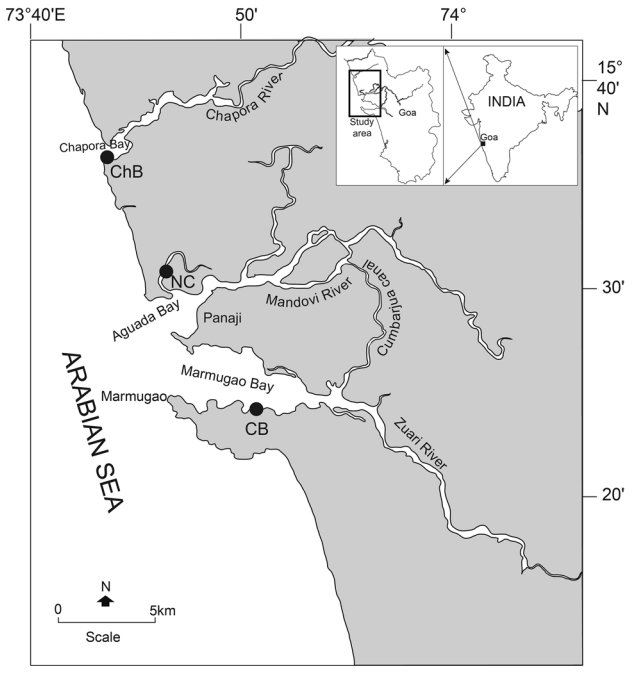

Fig. 1 Map showing sampling sites (CB: Chicalim Bay in Zuari estuary, NC: Nerul Creek in Mandovi estuary, ChB: Chapora Bay in Chapora estuary) along the Goa coast

During the low tide, sampling was carried out in the intertidal region of the above mentioned sites in monsoon (July 2013), post-monsoon (November 2013) and pre-monsoon (March 2014). Surface water temperature (°C) and pH were measured on site using a calibrated thermometer and a portable pH meter, respectively. Salinity (psu) was determined by a refractometer (ATAGO, S/Milli-E). Dissolved oxygen (DO) of surface water was analyzed using Wrinkler's method and expressed in mg l^{-1} (Parson et al. 1984). Oysters (n = 20 as one pool, 45–50 mm length) of each species from each site and season were collected and brought to the laboratory. Whole soft tissues of oyster were cleaned with deionized water to remove impurities. Then, tissues were dried at 60°C and digested with 65% HNO_3 (suprapure grade) as per the method described by Cheung and Wong (1992) with minor modification. Fish protein certified reference material for trace metals (DORM-4) was used to ensure the quality of the results. Accuracy of Cu, Ni, Cd and Pb analyses, are expressed in recovery percentage 87.23%, 87.83%, 98.03% and 71.46%, respectively. The precision measured as relative standard deviation (%RSD) of triplicate sample values were <10%. The detection limits for Cu, Ni, Cd and Pb were 100, 100, 10 and 50 µg kg^{-1}, respectively. Utmost care was taken at every step of sample processing to avoid contamination. The concentrations of metals (Cu, Ni, Cd and Pb) in all the samples were determined using a Graphite Furnace Atomic Absorption Spectrometer (Perkin Elmer, PinAAcle 900T).

The statistical analysis of data was conducted using PRIMER 6 (Primer-E Ltd., Plymouth, UK) software. Mean and standard deviation (SD) were calculated on all measured values of heavy metal concentrations. Data was also checked for normality (Shapiro-Wilks test) and homogeneity of variance (Levene's test). However, when data failed to meet normality, permutational MANOVA (PERMANOVA) test was performed on untransformed data to find out the significance level in spatio-temporal variation of metals concentrations. Finally, principle component analysis (PCA) was adopted to know the spatio-temporal relationship in metal concentrations measured in oyster tissue. Further, scatterplot on PCA scores of first two significant PCs were plotted across the axes to know the seasonal influence of a particular metal on the analyzed samples.

Results and Discussion

Metal uptake and accumulation in bivalves depend on several factors including size, sex, reproductive state, changes in tissue composition, bioavailability of metals, seasons and hydrodynamics of the environment (Boyden and Phillips

1981). In this perspective, physico-chemical properties such as temperature, pH, salinity and DO of surface water were measured at all the three sampling sites (Table 1). Considerably, low values of surface water temperature, pH and salinity and high values of DO were recorded in the monsoon period than in non-monsoon (pre-monsoon and post-monsoon). Dilution of seawater by heavy rainfall and influx of oxygen-rich riverine water together with cloud cover and low incoming solar radiation during monsoon could be the reasons for the observed lower surface water temperature, salinity and higher DO.

Table 2 summarizes the mean concentrations of Cu, Ni, Cd and Pb in the tissues of three oyster species from the three selected sites during three different seasons. The hierarchy of measured heavy metals content in the tissues of oysters was in the following order: Cu > Cd > Ni > Pb. The differences in heavy metals' burden in oysters may be due to the different affinity of metals to oyster tissues, different uptake, deposition and excretion rates. Moreover, results of PERMANOVA showed a significant variation ($p < 0.05$) in metals concentrations across all the seasons, sites and species as well as the interactions among these three main effects (Table 3). Although metals concentrations between study sites (CB and NC) and the reference site (ChB) showed significant ($p < 0.05$) variation, it is noteworthy that metal values observed at ChB was similar in range with CB and NC. This suggests that the reference site is also impacted by anthropogenic activities probably over the nearby fish landing jetty and sewage disposal from land inhabitants along the bank of the river Chapora. Similar hydrological parameters observed at all the three sites further supports the above statement. Metal concentrations in tissues of aquatic invertebrates living in the same habitat vary within closely related taxa and species within the same genus due to the species-specific differences for metal uptake and accumulation (Rainbow 2002 and references therein).

To understand the spatio-temporal relationship in the metal contents recorded in three different oyster species, a PCA analysis was performed (Fig. 2). Based on the eigenvalues (>1), the first two principal components (PCs) were retained as they explained 60.5% of the total variability. Loading values described the relationship between metal levels and the principal components. PC1 showed strong loadings of both Ni and Pb, while PC2 presented strong loadings of Cu and moderately high loading for Cd. This sets the stage for the seasonal groupings on the PCA scatterplot (PC1 vs. PC2). Three groups were formed which apparent with an emphasis on impacts among three different seasons irrespective of sites and oyster species. Notably, Group A (pre-monsoon) and Group C (monsoon) are separated largely on the basis of PC1. Group A was formed of six samples directed towards the upper right side of the plot showed signature of Ni and Cu. Group C composed of 5 samples located at the upper left side of a scatterplot showed less concentration of Pb and enrichment of Cd. On the basis of PC2, Group B (post-monsoon) comprised of eight samples, formed distinct group from Group A and C mainly due to low concentration of Cu and/or Cd. These results clearly demonstrate that different seasons and local conditions have more impact on accumulation of metals in oysters.

The concentrations of Cu in oyster tissues varied from 134.4 to 2167.9 mg kg^{-1} (Table 2), which is higher than the recommended limit (32 mg kg^{-1}) set by FAO Guideline (1983). The maximal levels of Cu in oyster tissues observed in pre-monsoon season is also supported by the Group A in the PCA scatterplot. The higher accumulation might be attributed to the increase in surrounding surface water temperature during pre-monsoon season. The rise in surrounding temperature increases the metabolic activities, resulting in higher filtration rate, larger collection of suspended matter and higher uptake of heavy metals (Belivermis et al. 2015). Another plausible reason could be due to the higher availability of Cu in the ambient environment. Since the studied sites are a famous destination for tourism and fishing, Cu-rich antifouling paint residues coming from barge building, fishing activities and tourism boats contaminates the estuarine environment (Alagarsamy 2006). Similarly, high values of Cu (170–610 mg kg^{-1}) were reported in tissue of *S. cucullata* from Deltaic Sundarbans (Sarkar et al. 1994). Since high concentrations of Cu (2100–4400 mg kg^{-1} dry wt.) cause oyster mortality (Hung and Han 1990), the excessive concentration of Cu noticed in the current study

Table 1 Seasonal variation in hydrological parameters from sampling sites

Parameters	Monsoon			Post-monsoon			Pre-monsoon		
	CB	NC	ChB	CB	NC	ChB	CB	NC	ChB
Water temperature (°C)	25	26	26	30	29	28.5	29	30	29.5
pH	6.75	6.23	6.25	8	7.7	8	7.9	7.8	8.1
Salinity (psu)	2	1	2	30	27	25	30	32	25
Dissolved oxygen (mg l^{-1})	7.41	5.62	6.12	6.58	5.80	5.71	5.47	4.39	5.36

CB Chicalim Bay, *NC* Nerul Creek, *ChB* Chapora Bay

Table 2 Seasonal variations in total metals (Cu, Ni, Cd and Pb) content (mean ± SD) (mg kg^{-1} dry weight) with % RSD (relative standard deviation) in three oyster species from selected sites

Metals	Seasons	Sites	*C. madrasensis*	%RSD	*C. gryphoides*	%RSD	*S. cucullata*	%RSD
Cu	Monsoon	CB	776.2 ± 0.3	0.49	485.4 ± 0.5	2.51	na	
		NC	na		na		638.9 ± 0.4	0.75
		ChB	359.4 ± 0.5	1.25	847.8 ± 0.2	0.33	na	
	Post-monsoon	CB	148.2 ± 0.2	1.68	542.4 ± 0.1	0.3	465.9 ± 0.3	0.86
		NC	383.2 ± 0.3	1.16	323.1 ± 0.7	2.79	134.4 ± 0.1	0.75
		ChB	275.1 ± 0.3	5.27	205.2 ± 0.5	2.83	255.3 ± 0.1	0.55
	Pre-monsoon	CB	606.8 ± 2.8	2.99	164.7 ± 0.1	0.41	1306.1 ± 4.1	4.02
		NC	1155.2 ± 2.8	3.23	526.1 ± 1.4	3.4	615.5 ± 1.4	1.41
		ChB	355.2 ± 2.1	1.89	na		2167.9 ± 1.8	2.65
Ni	Monsoon	CB	1.16 ± 0.3	2.22	0.88 ± 0.9	3.99	na	
		NC	na		na		1.43 ± 1.5	4.04
		ChB	2.06 ± 0.6	0.69	1.46 ± 0.0	1.26	na	
	Post-monsoon	CB	0.90 ± 0.5	4.39	1.56 ± 1.1	2.8	2.70 ± 0.4	1.04
		NC	1.24 ± 0.1	0.13	1.17 ± 1.4	0.09	0.70 ± 0.3	3.23
		ChB	1.66 ± 1.4	7.18	1.15 ± 1.1	3.77	0.84 ± 0.2	1
	Pre-monsoon	CB	4.51 ± 9.3	6.29	1.55 ± 0.3	1.38	0.88 ± 0.0	2.38
		NC	1.74 ± 0.2	4.78	3.18 ± 4.1	5.11	1.91 ± 0.4	8.05
		ChB	5.61 ± 0.2	2.77	na		2.96 ± 2.4	3.95
Cd	Monsoon	CB	154.6 ± 0.1	2.24	38.6 ± 0.2	3.45	na	
		NC	na		na		35.4 ± 0.0	0.3
		ChB	70.9 ± 0.1	1.15	88.5 ± 0.2	3.01	na	
	Post-monsoon	CB	9.5 ± 0.3	8.68	10.9 ± 0.1	1.88	11.7 ± 0.0	1.13
		NC	16.6 ± 0.3	2.48	20.2 ± 0.4	6.18	42.8 ± 0.4	7.36
		ChB	40.8 ± 0.1	5.15	18.0 ± 0.1	5.96	28.9 ± 0.1	2.89
	Pre-monsoon	CB	49.3 ± 0.0	0.51	7.5 ± 0.0	0.86	8.7 ± 0.0	0.59
		NC	21.1 ± 0.1	1.3	7.2 ± 0.1	1.8	9.3 ± 0.1	4.11
		ChB	19.3 ± 0.0	0.37	na		18.3 ± 0.0	0.77
Pb	Monsoon	CB	0.19 ± 0.5	5.28	0.19 ± 0.2	3.45	na	
		NC	na		na		0.20 ± 0.4	2.64
		ChB	0.35 ± 0.6	2.6	0.31 ± 0.3	1.13	na	
	Post-monsoon	CB	0.11 ± 0.3	4.38	0.35 ± 0.2	1.36	1.70 ± 0.1	1.33
		NC	0.44 ± 0.8	4.51	0.22 ± 0.2	2.71	0.12 ± 0.1	3.16
		ChB	0.84 ± 0.1	0.63	0.29 ± 0.1	0.76	0.32 ± 0.3	2.1
	Pre-monsoon	CB	0.68 ± 0.8	3.55	0.10 ± 0.0	0.91	0.13 ± 0.8	6.33
		NC	0.35 ± 0.1	0.17	0.22 ± 0.6	3.66	0.28 ± 2.0	9.5
		ChB	0.24 ± 0.3	1.81	na		0.35 ± 0.6	2.84

CB Chicalim Bay, *NC* Nerul Creek, *ChB* Chapora Bay, *na* species not available

indicates a higher risk to oysters in the Goa coast. Moreover, consumption of oyster tissue as seafood with such a high concentration of Cu can lead to stunted growth, cirrhosis of the liver and jaundice in humans (Gorman 1993).

The Ni concentrations in oysters varied between 0.70 and 5.61 mg kg^{-1} (Table 2). The maximum Ni value (5.61 mg kg^{-1}) was obtained in *C. madrasensis* tissue from ChB in pre-monsoon, while the minimum value (0.70 mg kg^{-1}) was measured in *S. cucullata* from NC in post-monsoon. The concentrations of Ni detected in this study were found well below the acceptable limit (70–80 mg kg^{-1}) (USFDA 1993). This shows that Ni does not have strong binding affinity in oyster tissue. Though the Ni concentration is far below the permissible limit, measured levels of Ni were comparatively high in pre-monsoon which was also statistically represented in Group A of the PCA scatterplot (Fig. 2). This tendency is hypothesized to be due to increase in filtration activity of oyster in non-monsoon period as discussed

Table 3 Results of PERMANOVA test for metal concentrations in relation to species, sites, seasons and their interaction

Source	df	SS	MS	Pseudo-F	*p* (perm)	perms
st	1	1661.3	1661.3	20683	0.001	998
se	2	19515	9757.3	1.21E+05	0.001	999
sp	1	497.24	497.24	6190.6	0.001	999
st × se	3	2715.5	905.16	11269	0.001	999
st × sp	4	10107	2526.8	31458	0.001	999
se × sp	3	7346.1	2448.7	30486	0.001	998
st × se × sp	4	12959	3239.8	40335	0.001	999
Residual	44	3.5342	8.03E-02			
Total	65	57153				

St site, *se* season, *sp* species, *df* degrees of freedom, *SS* sum of squares, *MS* mean sum of squares, *Pseudo-F* F value by permutation, *p* values are based on 999 permutations

earlier in case of Cu. A similar Ni level (5.67 mg kg^{-1}) was recorded in oyster *C. madrasensis* from Pulicat Lake (India) (Laxmi Priya et al. 2010).

The concentrations of Cd in oyster tissues ranged between 7.1 and 88.5 mg kg^{-1}. Since the concentration of Cd at Chicalim Bay (CB) was unexpectedly very high (154.6 mg kg^{-1}), it has been excluded from the range values. These values were observed to be much more elevated than the tolerable limit of 0.5 mg kg^{-1} (FAO 1983). Among four studied metals, Cd showed relatively high accumulation in monsoon than the non-monsoon period. These observations are coinciding with Group C of the PCA scatterplot. This could be attributed to three different reasons. First, although heavy rainfall during the monsoon period dilutes the metal concentrations in the aquatic system, a decrease in salinity (i.e. less chloride concentration) during the monsoon period increases the availability of free Cd ions to filter feeders (Engels and Fowler 1979) and thus higher metal uptake by oysters. Second, Cd mimics calcium (Ca) ions (which is abundant in seawater) due to its similar

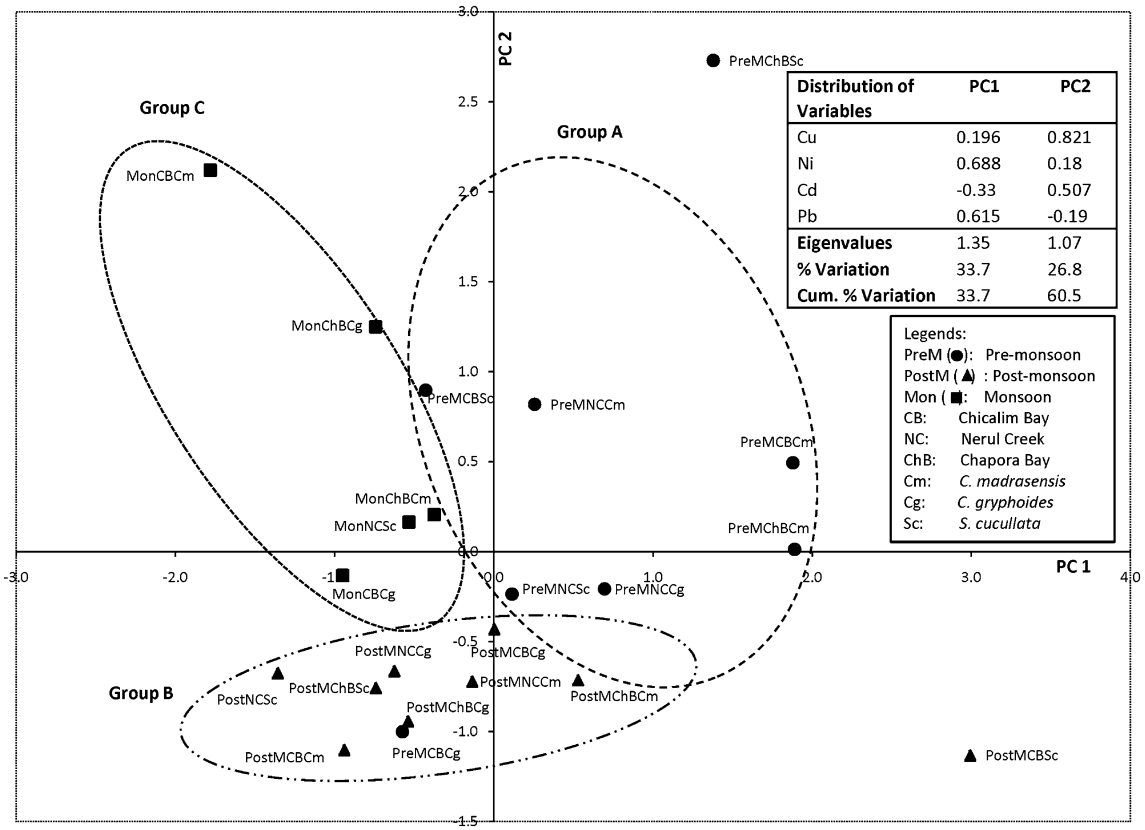

Fig. 2 The scatterplot of PCA scores (PC1 vs. PC2) of samples analyzed for metal concentrations in oyster species in three different seasons from selected sites

geochemical properties, particularly ionic radius (cf. Ca 9.7 and Cd 9.8 nm) (Huanxin et al. 2000). Third, Cd also has strong affinity towards the metallothionein (MT) proteins and sequestered metal in detoxified form like Cu (Rainbow 2002). Results of the present study is further corroborated by previous studies where authors have found high concentrations of Cd in clam *Paphia malabarica* (1.4–8.4 mg kg^{-1}) along the Goa coast (Kumari et al. 2006) and in oyster *S. cucullata* (10–40 mg kg^{-1}) along Deltaic Sunderbans (Sarkar et al. 1994). It has been found that Cd intakes of 0.43–0.71 µg kg^{-1} day^{-1} cause a toxic effect on consumers mainly to high risk groups, including women with low iron stores, people with renal impairment, smokers and children (Cheng and Gobas 2007). Therefore, consumption of oysters from the studied sites should be limited.

The highest value of Pb (1.70 mg kg^{-1}) was observed in *S. cucullata* in post-monsoon whereas, lowest value (0.10 mg kg^{-1}) was obtained in pre-monsoon season at CB (Table 2). Although the average concentration of Pb was below the recommended limit of 1.0 mg kg^{-1} (EU 2001), a few samples exhibited values >1.0 mg kg^{-1} which indicates that oysters from the study regions are contaminated by Pb to some extent. Heavy traffic load of motor vehicles, boats, ships, combustion of fossil fuel, organic waste discharge in the vicinity of sampling sites as reported by Veerasingam et al. (2015) could be the reasons for the Pb concentrations in some oyster samples.

Most metal toxicants could easily interchange and disperse through aquatic ecosystems. For example, re-suspension of surface sediment releases particulate matter along with metals into the overlying waters (Zvinowanda et al. 2009). Since oysters are filter feeders, it is necessary to further investigate the concentrations of bioavailable metals from sources like surficial sediment, particulate (suspended particulate matter) and dissolved metals from the oyster beds ambience.

Based on the results of our study we can conclude that waters along the Goa coast are highly contaminated with heavy metals. Furthermore, concentrations of Cu and Cd in oysters are above the limits recommended by international authorities for safe consumption by humans. Therefore, consumption of oysters from the studied sites should be avoided. It is important to identify source(s) of these metals and measure should be taken to reduce the metal pollution in seafood. The present work calls for continuous monitoring, people awareness and a stringent government policy to control metal pollution in the coastal waters along the Goa coast.

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Antioxidant responses in gills and digestive gland of oyster *Crassostrea madrasensis* (Preston) under lead exposure

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ABSTRACT

Crassostrea are ecologically and economically important bivalves and provide a good livelihood for coastal regions of many countries, including India. This study aims at evaluating the response of the antioxidant defense system in oyster *Crassostrea madrasensis* against lead (Pb) exposure under laboratory conditions. Antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione-s-transferase (GST) and oxidative damage parameter lipid peroxidation (LPO) were measured in the gills and digestive glands of oysters exposed to 1–50 μg/l of Pb $(NO_3)_2$ over a period of 8 days. LPO index increased progressively with increase in Pb concentration (1, 10, 25 and 50 μg/l) in both tissues, gills and digestive gland. Although CAT and SOD activities induced together in the initial phase (upto 6th day), their activities decreased at a later stage of the experiment. However, GST activity increased on 8th day in both the tissues at concentration 10, 25 and 50 μg/l indicates the compensatory defense mechanism against oxidative stress. The induced antioxidant responses recorded at 25 and 50 μg/l of Pb concentrations suggest the presence of Pb-induced oxidative stress at these concentrations. The results of this work also indicate that LPO, SOD, and GST could be used as biomarkers to assess the impact of Pb on the *C. madrasensis*. This study concludes that any high level of dissolved Pb concentration (> 10 μg/l) in surrounding seawater could be harmful to the physiology of the *C. madrasensis*.

1. Introduction

Oysters (Mollusca: Bivalvia) are sedentary, known to bioaccumulate and tolerate high concentrations of metals (several magnitudes higher than their ambient concentrations). Therefore, worldwide they are used extensively as a biomonitor for metal pollution and eco-toxicological studies (O'Connor, 2001; Funes et al., 2006; Alfonso et al., 2013). Oysters are popular worldwide due to their high nutritional values (Asha et al., 2014). In India, 2400 t of oyster production was recorded from the Indian coast in the year 2008 (National Aquaculture Sector Overview- India).

Lead (Pb) is ubiquitous in presence and one of the most toxic element occurs in almost all the ecosystem (Hsu and Guo, 2002; Flora et al., 2012). It is derived from both natural sources such as weathered (dissolved) or eroded (particulate) earth's surface, volcanic activities, atmospheric aerosol, biogenic forest fires as well as anthropogenic activities. In recent years, however, increase in various anthropogenic activities such as lead smelting, use of lead based-antifouling paints, ceramics, plastics, leaded gasoline, combustion of fossil fuel have significantly increased the Pb concentration in the aquatic environment (MacFarlane, 2001; Flora et al., 2012). High to moderate levels of Pb content have been found in tissues of edible clam *Paphia malabarica* (average: 30.3 mg/kg) (Krishna Kumari et al., 2006), suspended particulate matter (range: 27.6–91.4 mg/kg) (Kessarkar et al., 2013) and in surface sediments (average: 22.6 mg/kg) (Veerasingam et al., 2015) across the estuarine waters of Goa. Moreover, higher concentrations of Pb level (> 1 mg/kg, permissible limit) have been observed in the tissues of edible oyster *Crassostrea* sp. from the coastal waters of Goa (Shenai-Tirodkar et al., 2016). It is recognized that impact of these accumulated metals cannot be judged only by quantification because it does not provide a clear indication of toxic effects of pollutant on the aquatic organism (Livingstone, 2001).

Biochemical biomarkers have been commonly used to measure the effects of contaminants on organisms in ecotoxicological studies (Livingstone, 1993). Under normal metabolic processes, a balance between generation and neutralization of reactive oxygen species (ROS) include superoxide anion radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\bullet}HO$) is maintained by organisms. However, it has been proved that bioaccumulated metal ions enhance the formation of reactive radicals in the cells which cause the cell damage (Leonard et al., 2004; Valko et al., 2005). It is well known that exposure to Pb boosts the ROS production, which in turn results in damage to the

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cell membrane, protein oxidation, lipid peroxidation and DNA damage (Halliwell and Gutteridge, 1989; Gurer and Ercal, 2000; Ercal et al., 2001; Hsu and Guo, 2002; Flora et al., 2012). This oxidative damage can be assessed by measuring thiobarbituric acid reactive substances (TBARS) reflecting the state of lipid peroxidation of the cell membrane (Ohkawa et al., 1979). Further, to protect against oxidative stress organism possess major antioxidant defense system which includes three main enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), and glutathione-s-transferase (GST). However, noticeable variation in responses (induction and/or inhibition) of biochemical biomarker were recorded in previous laboratory and field studies which mostly depend on the type and amount of contaminants, type of species, the tissue, and the sampling time, etc. (Zanette et al., 2006; Luna-Acosta et al., 2010; Cotou et al., 2013).

Presently, there is very limited work published with respect to Pb exposure in bivalves (Prakash and Jagannatha Rao, 1995; Yan et al., 1997; Dafre et al., 2004; Fernandez et al., 2010). Particularly, Pb toxicity study on oyster species from Indian coast has not been conducted which is harvested and consumed on large scale and on regular basis. Exposure of oysters to various concentrations of Pb, coming from discharge of dissolved and particulate forms of Pb into the marine ecosystem of Goa coast (India), has necessitated this study to understand the antioxidant defense response of oysters against Pb exposure. It has been proved that field-based effects could well be understood by conducting laboratory exposures of organisms to a particular contaminant (Verlecar et al., 2008).

In this study, gills and digestive glands of oyster, *C. madrasensis*, were examined to assess the effect of Pb exposure using biochemical markers such as LPO, SOD, CAT and GST. The reason for selecting gills and digestive gland is because these tissues form an active site for xenobiotic uptake and oxy-radical generation as well as enzyme biotransformation process (Livingstone et al., 1992).

2. Materials and methods

2.1. Sampling site and oyster collection

A total of 150 individual adult oysters, *C. madrasensis* (60–70 mm), irrespective of sex, were collected during low tide in post-monsoon (October 2015) from Galgibag estuary, South Goa coast, India. Galgibag site (Lat. 14°57' 54.66"N Long. 74°03'20.16"E) was chosen because this site is located far away from potential pollution sources such as industries and harbor activities (Fig. 1). The hydrological parameters (water temperature: 29 ± 1 °C, pH: 7.9 ± 0.4 and salinity: 27 ± 1 psu) were recorded at the time of oyster collection. The samples were brought to laboratory in seawater and cleaned to remove fouling organisms and debris. All the oysters were acclimatized for 8 days in aerated seawater at room temperature before conducting the acute semi-static renewal Pb toxicity (8 days) experiment (Thompson et al., 2012).

2.2. Experimental design

After the acclimatization period, the oysters were distributed into five different groups (n = 15, each tank) and subjected to five treatments: one seawater control and four different Pb concentrations (1 µg/l, 10 µg/l, 25 µg/l, and 50 µg/l). These concentrations were selected based on environmental realistic concentration (Shenai-Tirodkar et al., 2016) and concentration large enough to induce biomarker response. Mortality was monitored and dead oysters were removed. Under these conditions, the state of the antioxidant enzyme system was assessed.

The experiment was setup in duplicate in 25 l capacity plastic tanks (~1.5 l of seawater per oyster). Seawater was continuously aerated and standard hydrological parameters (temperature 29 ± 1 °C, pH 8.2 ± 0.4 and salinity 27 ± 1 psu) were recorded throughout the experiment. Lead nitrate [Pb $(NO_3)_2$] (Merck India Ltd, Mumbai) was

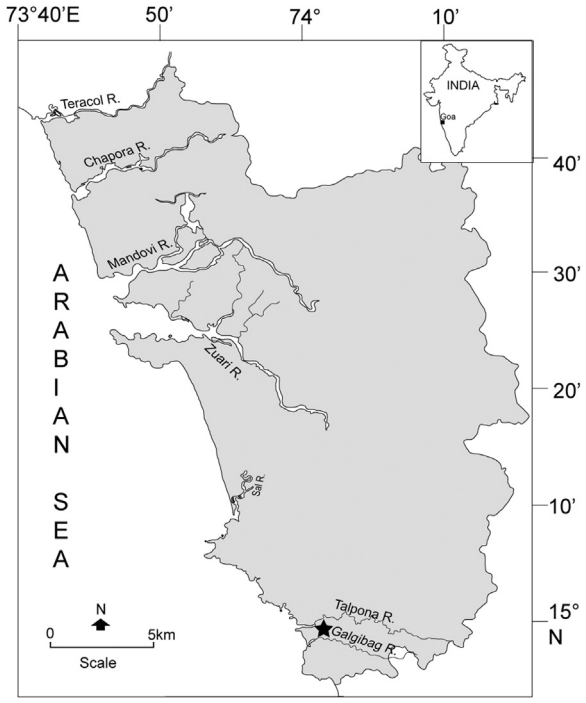

Fig. 1. The location of sampling site (⋆) at Galgibag estuary from Goa coast.

used to prepare stock solution (100 mg/l) and preserved in a refrigerator until use. This was treated as main stock solution from which fresh sub-stock solutions were prepared and used for preparation of required concentrations of Pb. All the glassware used during experiment was washed with 10% HNO_3 (nitric acid) and well rinsed with distilled water. The whole exposure media was renewed on daily basis and replaced with fresh natural seawater, maintaining respective concentrations of Pb $(NO_3)_2$. Oysters were not fed during experimentation. At each sampling (2nd, 4th, 6th and 8th day of experiment duration), two of the surviving oysters were sacrificed from each treatment tank and immediately gills and digestive gland were dissected for further analysis. All the further analyses were performed in triplicates to rule out the experimental bias.

2.3. Determination of Pb content in gills and digestive gland of *C. madrasensis*

Gills and digestive gland of *C. madrasensis* were processed for Pb estimation according to the method described in Cheung and Wong (1992) with slight modifications. In detail, both the tissues were dried at 60 °C till constant weight was attained, and ground by using agate mortar and pestle. The 0.5 g of dried tissue powder was soaked overnight in 5 ml of 65% HNO_3. Reaction mixture was heated at 80 °C till dry and re-dissolved in 3 ml of 65% HNO_3 to achieve the complete digestion of the tissue. The digest was allowed to cool at room temperature. The digested clear solution was then filtered through Whatman No. 42 filter paper and diluted to 10 ml with 2% HNO_3 used for metal determination. Fish protein certified reference material (DORM–4) was used to ensure the quality of the results. Determination of Pb was made by using Flame Atomic Absorption Spectroscopy (Flame-AAS). Utmost care was taken at every step of processing the sample, to avoid contamination. All reagents used in the analysis were of the purest grade. Appropriate blanks and standards were also prepared by using the same method. Appropriate dilutions of samples were made depending on the sensitivity of detection in these samples. Precision measured as relative standard deviation (% RSD) of triplicate sample values were < 10%. Accuracy of Pb analysis was expressed in recovery percentage 91.35%.

2.4. Biochemical analysis of gills and digestive gland of C. madrasensis

2.4.1. Sample preparation

The gills and digestive gland of *C. madrasensis* were thoroughly washed with phosphate buffer (50 mM; pH 7.4) and homogenized in 50 mM phosphate buffer (1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), 0.15 M potassium chloride (KCl), 0.01% phenylmethylsufonyl fluoride (PMSF), pH 7.4). Homogenization was carried out using motor driven Teflon Potter-Elvejhen homogenizer. Further, homogenates were centrifuged at 10000 × *g* for 30 min at 4 °C. The resulting supernatant fractions were stored at −80 °C until the quantification of the activities of LPO, SOD, CAT, and GST.

2.4.2. Estimation of lipid peroxidation

The LPO index was estimated using the protocol described by Ohkawa et al. (1979) with minor modifications. Briefly, tissue homogenate (0.5 ml) was mixed with 1.5 ml of 0.8% thiobarbituric acid (TBA) solution, 1.5 ml of 20% acetic acid (pH 3.5), 0.2 ml of 8.1% sodium dodecyl sulfate (SDS), 0.2 ml double distilled water and 0.1 ml of 0.76% butylated hydroxytoluene (BHT). The mixture was heated at 95 °C for 60 min and cooled at room temperature. After that samples were centrifuged at 5000 × *g* for 10 min and the absorbance of the supernatant was measured at 532 nm against blank. The LPO index was calculated by using an extinction coefficient of 1.56 × 105/M/cm (Wills, 1969) and expressed as nmol TBARS formed/mg protein.

2.4.3. Estimation of superoxide dismutase and catalase activity

Superoxide dismutase (EC 1.15.1.1) and catalase (EC 1.11.1.6) activities were measured by using EnzyChrom superoxide dismutase assay kit (ESOD-100) and EnzyChrom catalase assay kit (ECAT-100) respectively, as per the manufacturer's instructions. Both the kits were bought from Bioassay Systems, USA. Assays were performed in a microplate reader (FLUOstar Omega; BMG LABTECH Pty. Ltd.) by using 96 well plates. The results of SOD were expressed as Unit/mg protein whereas CAT activity was presented as nkat/mg protein.

2.4.4. Estimation of glutathione s-transferase activity

Glutathione-s-transferase (EC 2.5.1.18) activity was determined following the methods of Mannervik (1985) with minor modifications. The reaction mixture contained 950 µl assay buffer phosphate-buffered saline (PBS) (pH 6.5), 10 µl of 100 mM glutathione (GSH), 30 µl sample (200–250 µg protein) and 10 µl of 100 mM 2,4- dinitrochlorobenzene (CDNB). The difference in absorbance was measured at 340 nm for every 30 s for 5 min. The activity of GST was determined using molar extinction coefficient of 0.0096/µM/cm for CDNB. The results were expressed as nmol CDNB conjugates formed/min/mg protein.

2.4.5. Estimation of total protein concentration

Total protein concentrations of tissue samples were analyzed using bovine serum albumin (BSA) as standard as described by Lowry et al. (1951).

2.5. Statistical analysis

Data were expressed as mean ± standard deviation (SD) (n = 3). All the data were first checked for normality using Shapiro–Wilk's test, and homogeneity of variance by using Levene's test. Data that failed to meet the normal distribution were transformed by log (x + 1) transformation prior to statistical testing. Significance of variability among groups with Pb concentrations, exposure time and the tissues were evaluated for each biomarker as well as for the Pb content by using three-way analysis of variance (three-way ANOVA). For the multiple comparison among groups post hoc test (Tukey's HSD test) was performed. To find out the significant relationship, Pearson correlations were performed among Pb content, oxidative stress and antioxidant response in gills and digestive glands of *C. madrasensis*. Statistical significance of data was measured at $P < 0.05$ level. All the tests were performed using STATISTICA 8 (StatSoft, Inc., USA) software.

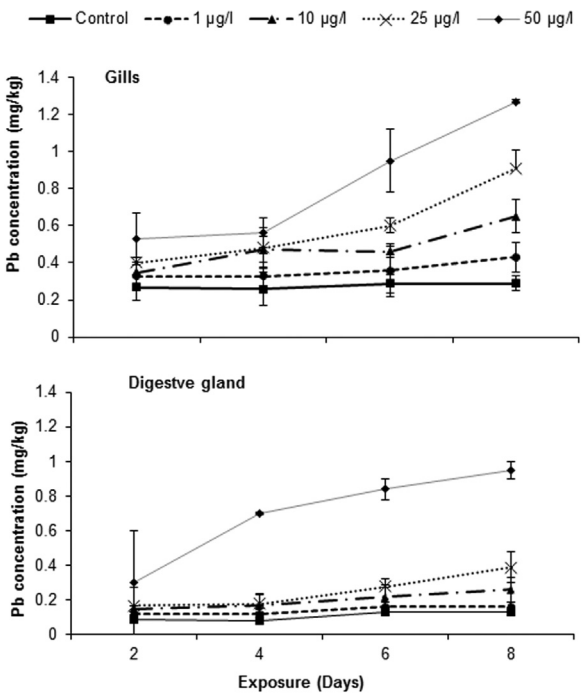

Fig. 2. Total average Pb concentrations (mg/kg) ± SD (n = 3) in gills and digestive gland of control and exposed *C. madrasensis* to various concentrations of Pb $(NO_3)_2$ for 8 days.

3. Results

During acclimatization period, dead oysters were removed from the respective tanks. After acclimatization, during the experimental period, oyster's mortality was observed only in tanks containing concentration of Pb more than 10 µg Pb/l. A total of 3 oysters died from 10 µg Pb/l treated tank on 5th and 7th day. Similarly, 3–4 oysters died at 25 µg Pb/l and 50 µg Pb/l treated tank on 4th and 7th day respectively.

3.1. Concentration of Pb in gills and digestive gland of C. madrasensis

Concentrations of Pb in gills ranged from 0.27 ± 0.07 mg/kg to 1.27 ± 0.01 mg/kg whereas in digestive gland, it varied between 0.1 ± 0.05 mg/kg and 0.95 ± 0.05 mg/kg (Fig. 2). On the 8th day, 50 µg/l treated oysters showed the highest accumulation (1.27 ± 0.01 mg/kg) in gills which is more than the allowable limit for human consumption according to guidelines set by EU (2001). Accumulation of Pb in the gills and digestive gland of *C. madrasensis* increased significantly ($p < 0.05$) over a period of days in each Pb treatments as compared to control. Further, significantly higher accumulation of Pb was observed in gills than digestive gland during the experiment at all the exposed concentrations (Table 1).

3.2. Biomarker responses in gills and digestive glands of C. madrasensis

Oxidative stress indices such as LPO, SOD, CAT, and GST in gills and digestive gland of oyster have been measured under the semi-static Pb exposure experiment. Considerable changes took place in the oxidative defense system of gills and digestive gland after exposure to different concentration. Results of three-way ANOVA revealed the significant ($p < 0.05$) differences in biomarker responses among variables such as concentrations, exposure time (days), types of tissue and interactions among these variables (Table 2).

Table 1
Results of three-way ANOVA on the Pb concentrations in gills and digestive glands of *C. madrasensis* under control and Pb $(NO_3)_2$ treated conditions (1 μg/l, 10 μg/l, 25 μg/l, 50 μg/l) during 8 days experiment.

Variables	SS	*df*	MS	F	*p* value
Day	1.26266	3	0.42089	56.256	*0.000*
Concentration	4.82320	4	1.20580	161.167	*0.000*
Tissue	1.57094	1	1.57094	209.972	*0.000*
Days × Concentration	1.01723	12	0.08477	11.330	*0.000*
Days × Tissue	0.11394	3	0.03798	5.076	*0.003*
Concentration × Tissue	0.17656	4	0.04414	5.900	*0.000*
Days × Concentration × Tissue	0.16915	12	0.01410	1.884	*0.049*

df: degrees of freedom, SS: sum of squares, MS: mean sum of squares. *Italic* values denotes the significant level at $p < 0.05$.

3.2.1. Lipid peroxidation (LPO)

LPO index in both gills and digestive gland showed significant ($p < 0.001$) increase against each Pb treatment as compared to respective control. Moreover, LPO index showed strong positive correlation with the Pb content ($p < 0.001$) in gills and digestive gland (Table 3). Although it was observed that continuous oxidative load is experienced by gills, digestive gland exhibited significantly higher oxidative stress compared to gills on 2nd and 6th day (Fig. 3a and b). Further, the digestive gland of oysters exposed to 50 μg/l showed declined LPO activity significantly from 6th day to 8th day ($p < 0.001$).

3.2.2. Superoxide dismutase (SOD)

Fig. 3(c and d) and Table 2 shows that SOD activity in digestive gland was significantly higher than gills. SOD activity in gills and digestive gland from control tank did not show much variation during successive exposure periods. In particular, gills showed significant ($p < 0.001$) increase in SOD activity at 10, 25 and 50 μg/l treated groups until 6th day, which then dropped prominently ($p < 0.001$) on 8th day (except 10 μg/l). A similar trend was observed in digestive gland from all the treated groups (except in 50 μg/l treated group where SOD activity significantly increased). Activities of SOD was closely correlated with Pb concentrations and LPO index in both the tissues (Table 3).

3.2.3. Catalase (CAT)

Activity of CAT in gills increased significantly ($p < 0.001$) until the 4th day in 1 and 10 μg Pb/l exposed groups and till the 6th day in 25 and 50 μg/l exposed groups. This was followed by a slight decrease in CAT values on 8th day. Whereas gills from control group showed no significant variation in CAT measurements throughout the experiment. A similar trend of CAT activities was also noticed in digestive gland of treated oysters ($p < 0.001$) (Fig. 3e and f). The CAT activity showed a positive correlation with Pb concentration, LPO ($p < 0.01$) and GST ($p < 0.05$) in both gills and digestive gland of oyster (Table 3).

3.2.4. Glutathione S-transferases (GST)

Response of GST in gills and digestive gland to Pb exposure are shown in Fig. 3g and h. Low levels of GST was observed in gills and digestive gland ($p < 0.05$) from all Pb exposed groups compared to controls till the 6th day (except in 50 μg/l). However, on the 8th day, GST levels in gills increased in 10, 25 and 50 μg/l exposed groups while in digestive gland it increased in all the exposed groups (1, 10, 25 and 50 μg/l). Activities of GST in gills was positively correlated with Pb concentration, LPO and CAT whereas Pb concentration, LPO and SOD showed positive correlation in digestive gland (Table 3).

4. Discussion

Under metal exposure, oyster accumulates metal at very high concentrations in different body parts to different extents (Marigomez et al., 2002) which generates several biological responses depending on metal concentration and duration of exposure (Hariharan et al., 2014).

Table 2
Results of three-way ANOVA on the biomarker responses measured in gills and digestive gland of *C. madrasensis* under control and Pb $(NO_3)_2$ (1 μg/l, 10 μg/l, 25 μg/l, 50 μg/l) treated conditions during experimental period.

		SS	*df*	MS	F	*p* value
LPO	Day	0.41706	3	0.13902	1237.8	*0.000*
	Concentration	1.54713	4	0.38678	3443.8	*0.000*
	Tissue	11.71074	1	11.71074	104270.2	*0.000*
	Day × concentration	0.08256	12	0.00688	61.3	*0.000*
	Day × tissue	0.01834	3	0.00611	54.4	*0.000*
	Concentration × tissue	0.37175	4	0.09294	827.5	*0.000*
	Day × concentration × tissue	0.08289	12	0.00691	61.5	*0.000*
SOD	Day	63.72	3	21.24	13.016	*0.000*
	Concentration	887.94	4	221.99	136.046	*0.000*
	Tissue	11166.56	1	11166.56	6843.508	*0.000*
	Day × concentration	73.51	12	6.13	3.754	*0.000*
	Day × tissue	61.19	3	20.40	12.500	*0.000*
	Concentration × tissue	853.18	4	213.30	130.720	*0.000*
	Day × concentration × tissue	72.99	12	6.08	3.727	*0.000*
CAT	Day	2724	3	908	111.7	*0.000*
	Concentration	3330	4	832	102.4	*0.000*
	Tissue	88	1	88	10.8	*0.002*
	Day × concentration	4662	12	389	47.8	*0.000*
	Day × tissue	128	3	43	5.3	*0.002*
	Concentration × tissue	394	4	98	12.1	*0.000*
	Day × concentration × tissue	2222	12	185	22.8	*0.000*
GST	Day	0.6502	3	0.2167	106.1	*0.000*
	Concentration	0.4196	4	0.1049	51.4	*0.000*
	Tissue	0.1014	1	0.1014	49.7	*0.000*
	Day × concentration	0.6426	12	0.0535	26.2	*0.000*
	Day × tissue	0.0513	3	0.0171	8.4	*0.000*
	Concentration × tissue	0.6018	4	0.1504	73.6	*0.000*
	Day × concentration × tissue	1.0891	12	0.0908	44.4	0.000

df: degrees of freedom, SS: sum of squares, MS: mean sum of squares. *Italic* values denotes the significant level at $p < 0.05$.

Table 3
Correlation (Pearson) between Pb accumulation and various antioxidant parameters in gills and digestive gland from control and exposed *C. madrasensis*.

	Gills					Digestive gland				
	Pb content	LPO	SOD	CAT	GST	Pb content	LPO	SOD	CAT	GST
Pb content	1.00					1.00				
LPO	**0.91**^{c}	1.00				**0.91**^{c}	1.00			
SOD	**0.71**^{c}	**0.72**^{c}	1.00			**0.81**^{c}	**0.76**^{c}	1.00		
CAT	**0.59**^{b}	**0.58**^{b}	**0.53**^{a}	1.00		**0.67**^{b}	**0.68**^{b}	**0.61**^{b}	1.00	
GST	**0.77**^{c}	**0.68**^{b}	0.36	**0.47**^{a}	1.00	**0.79**^{c}	**0.70**^{b}	**0.53**^{a}	0.42	1.00

Significance level at $p < 0.05$ = a, $p < 0.01$ = b, $p < 0.001$ = c.

In the present experimental set up mortality of oysters were observed at the higher concentrations (10, 25 and 50 µg/l) of Pb exposed group. This could be due to increase in the levels of intracellular toxic metabolites. Moreover, accumulations of Pb in live oyster's gills was found to be approximately 2–4 times higher than in the digestive gland. The reasons for the higher concentrations in gills may be due to its continuous exposure to the surroundings and the large surface area of thin epithelium (Soldatov et al., 2007; Fernandez et al., 2010). Similar to these results, Prakash and Jagannatha Rao (1995) also recorded high metal accumulation in gills than digestive gland of mussel *Perna viridis*.

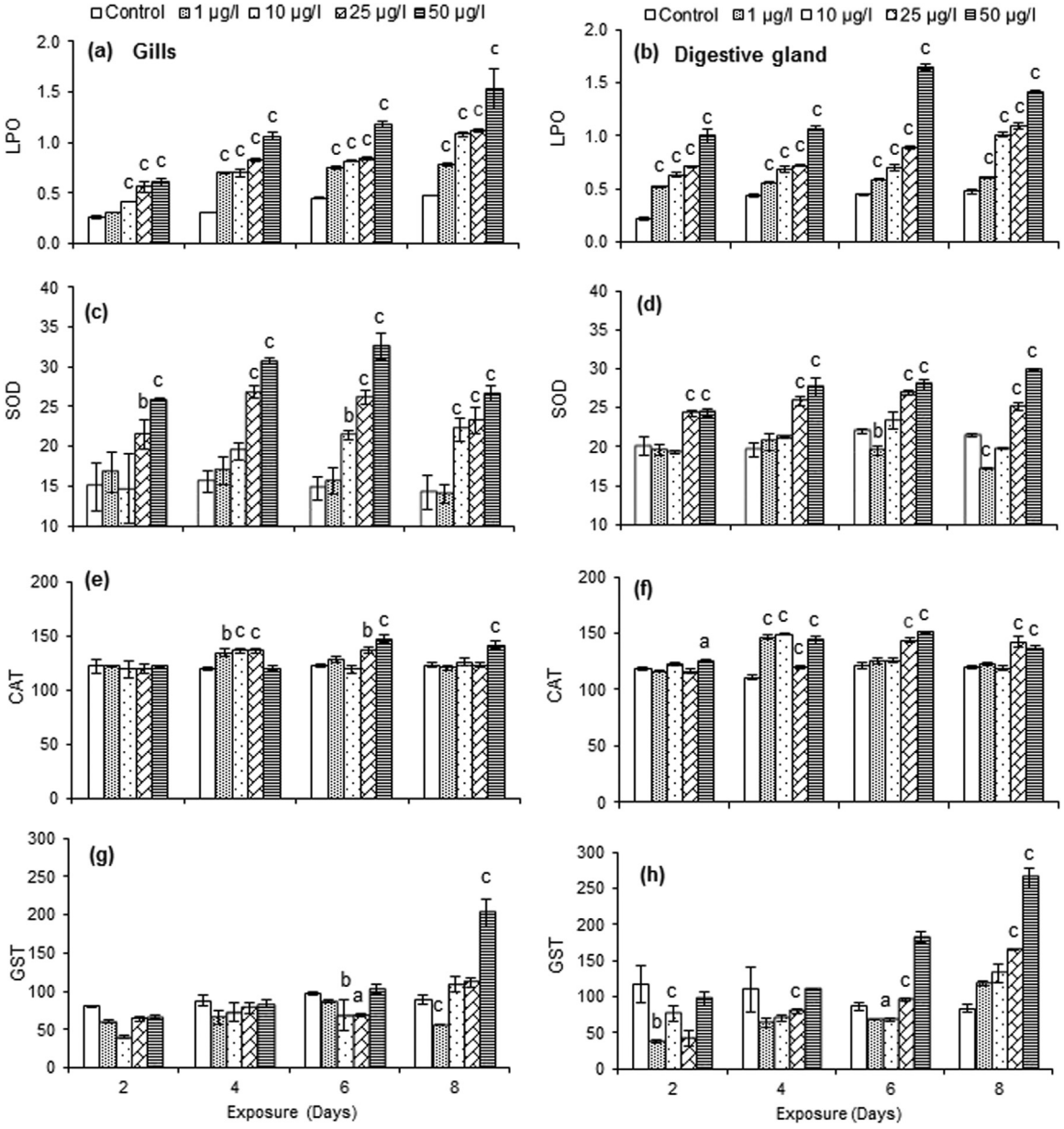

Fig. 3. Effect of Pb on (a and b) lipid peroxidation (nmol TBARS formed/mg protein), (c and d) superoxide dismutase (Unit/mg protein), (e and f) catalase (nkat/mg protein), (g and h) glutathione-s-transferase (nmol CDNB conjugate formed/min/mg protein) in gills and digestive gland of *C. madrasensis*. Data are expressed as average ± SD (n = 3). Significant differences between treated group and control group denotes with superscript letter a, b and c at $p < 0.05$, $p < 0.01$ and $p < 0.001$ level respectively.

Alcutt and Pinto (1994) observed Pb equilibrium between tissue of clam, *Mercenaria mercenaria* and surrounding aquatic medium within short exposure (five days). The high accumulation of Pb in tissues of bivalves occurs due to the low excretion rates of amorphous granules and their immobilization in the shell (Viarengo, 1989).

In the current study, TBARS product was prominently increased in gills and digestive gland in all Pb exposed groups compared to unexposed (control) group. Significant and positive correlation ($p < 0.001$) was found between LPO and Pb concentration in both the tissues (Table 3). This infers that oxidative stress increases as a consequences of rise in ROS formation in lipid membranes due to gradual increase in Pb accumulation (Stohs and Bagchi, 1995; Ercal et al., 2001). It is known that marine organisms that are rich in polyunsaturated fatty acids increase ROS generation during metal exposure (Liu et al., 1997). This can lead to an imbalance in pro-oxidant and antioxidant processes, enhance oxidative damage and generate TBARS product (Manduzio et al., 2005). Another reason for high level of TBARS induction in both the tissues from exposed groups compared to control group may be due to the structural and functional characteristics of both the organs. Firstly, It is important to point out that continuous oxidative load was experienced by gills in this study. As discussed earlier, an oyster's gill structure may be the reason that it showed more effect of Pb stress in successive concentrations (Soldatov et al., 2007). Secondly, since the digestive gland is the main organ involved in metabolic function, stress induced from various Pb concentrations may directly affect the metabolic pathway, resulting in both increased production and accumulation of ROS (Livingstone, 1992; Trombini et al., 2010). Consequences of these generated free radicals damages the membrane phospholipids and also change in fatty acid composition of molecules (Knowles and Donaldson, 1990; Hsu and Guo, 2002). This oxidative stress can further bring changes in the antioxidant enzyme activities (Manduzio et al., 2005; Gonzalez et al., 2015). The results of present study are corroborated by the findings of previous studies where researchers have found increased LPO in metal toxicity in mussels (Prakash and Jagannatha Rao, 1995; de Almeida et al., 2004; Vlahogianni and Valavanidis, 2007; Tsangaris et al., 2010) and prawn *Penaeus monodon* (Hariharan et al., 2012).

Superoxide dismutase is the first and crucial antioxidant enzyme which removes the superoxide radical through the process of dismutation to oxygen and hydrogen peroxide ($2O_2^{\bullet -} + H^+ \rightarrow H_2O_2 + O_2$) (Fridovich, 1975). It prevents generation of highly toxic $^{\bullet}OH$ radicals. In the present study, SOD activity was slightly more in the digestive gland than gills. Moreover, positive correlation ($p < 0.001$) was found between the SOD and LPO as well as Pb concentration in both the tissues (Table 3). The observed positive correlation indicates that response of SOD was linked to Pb accumulation and its generated oxidative stress. SOD increased noticeably until the 6th day of exposure and decreased slightly on the 8th day in both the organs (prominently in gills). The initial increase in the SOD suggests the protective behavior of the cell against $O_2^{\bullet -}$ radicals produced by activation of the SOD initially while its depletion later on indicates the probable SOD degradation by ROS or its over utilization to overcome with Pb toxicity (Dafre et al., 2004). Furthermore, SOD activities in gills dropped more rapidly than those of the digestive gland suggesting that the detoxification function of digestive gland is more effective than the gills to counteract the high LPO level generated under plausible Pb toxicity (Trombini et al., 2010). This results are in the accordance with previous studies carried out by Jing et al. (2007) on oyster *Pinctada fucata* where SOD was decreased gradually in later part of Pb exposure experiment. Dai et al. (2012) were also recorded the inhibition of SOD in tilapia (*Oreochromis niloticus*) during dietary Pb exposure.

Catalase is a ubiquitous oxidoreductase antioxidant enzyme that breaks down the hydrogen peroxide molecule to water and oxygen molecules ($2H_2O_2 \rightarrow 2H_2O + O_2$) and thus removes the toxicity of H_2O_2 (Livingstone, 2001). In the present study, significant positive correlation ($p < 0.01$) was detected between CAT and Pb concentrations, LPO, SOD which suggest that SOD and CAT function together in a coordinated manner and neutralizes the generation H_2O_2, against the oxidative stress induced by Pb (Jena et al., 2009). Moreover, like SOD, CAT levels in both the tissues (gills and digestive gland) increased until the 4th and 6th day and declined slightly in the later phase of the experiment. These reductions in CAT impairs scavenging of H_2O_2 radical leading to the enhancement of LPO in both the tissues (Ercal et al., 2001). Similar results were obtained in green mussel *Perna viridis* under Pb exposure (Hariharan et al., 2014). According to our results, low activities of SOD and CAT indicate a non-significant contribution of these enzymes to antioxidant defense processes in the later phase of the experiment, which further may be associated with difficulty in compensating oxidative stress. Similarly, scientists have observed that CAT and SOD target Pb by forming the complexes with their substrates which is responsible for inhibition of these enzymes and deactivating them (Ercal et al., 2001; Marques et al., 2016). This decrease in SOD and CAT may results in increase in $O_2^{\bullet -}$ radicals accumulation with combination of H_2O_2 generate hydroxyl $^{\bullet}OH$ radicals (under classic Haber–Weiss reaction) resulting in rise of TBARS substances (Verlecar et al., 2008).

Glutathione-s-transferase is a major phase II-metabolic enzyme which plays a major role in detoxification of both xenobiotics and oxidative metabolic by-products (Van der Oost et al., 2003; Jozefczak et al., 2012). In the present study, gills and digestive gland showed initially less GST response which was below control level but it increased progressively from the 6th day onwards, indicating activation of detoxification mechanism in the gills as well as digestive gland cells. Apparently, the GST did not play an important role in neutralization of oxidative load initially, as judged by the low level of GST. Moreover, compensatory adaptive mechanisms between different biochemical biomarkers may occur (Regoli and Principato, 1995). Significant increase in GST activities in the later phase (8th day) of the experiment demonstrates the protective action of GST against reactive oxygen radicals to reduce Pb toxicity. This is also supported by the Pearson's correlation results where GST is positively correlated with the Pb concentration ($p < 0.001$), LPO ($p < 0.01$). This increase in GST activities may be a compensatory adaptive mechanism to neutralize increased levels of ROS when SOD and CAT were depleted from the 4th day onwards (Fernandez et al., 2010). Furthermore, digestive gland showed more GST activities as compared to gills. This elucidates that the gills are weak in detoxification process in the oxidative defense system as mentioned earlier. These results also corroborate the findings of Yan et al. (1997) where the authors found an effective increase in the glutathione content in the gills and digestive gland of green mussel *Perna viridis* after ~21 days of exposure to Pb stress. Similarly, Dafre et al. (2004) observed an increase in glutathione level against the Pb stress in brown mussel *Perna perna*. In contrast, some researchers have found that Pb inactivates glutathione by binding it to sulfhydryl group present in it, which further depresses the glutathione level (Ahamed and Siddiqui, 2007). Thus, measurement of a single antioxidant is not enough to state a complete antioxidant defense under laboratory as well as field conditions. On the whole, our study shows that Pb generate the oxidative stress in gills and digestive gland and induces activation of SOD and CAT in the initial phase, whereas GST takes a position as a compensatory of the defense system in later stages. In this experiment, Pb toxicity arises may be due to two different, although related pathways: first, the ROS formation which includes $O_2^{\bullet -}$, H_2O_2 and $^{\bullet}HO$ and second, the depletion of antioxidant reserves (Flora et al., 2002).

5. Conclusion

Based on the results of the investigated biochemical biomarkers LPO, SOD, CAT and GST we conclude that oxidative stress generated in gills and digestive gland of *C. madrasensis* is specifically depend on the concentrations and duration of Pb exposure. Furthermore, acute to high

levels (≥10 μg/l) of dissolved Pb in surrounding seawater could be harmful to the physiology of the oyster. However, in future, similar studies should be conducted for longer exposure periods and with other biomarkers to present a complete overview of mechanisms of oxidative stress regulation in this sentinel species.

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