CHAPTER 2

MATERIALS AND METHODS
2.1. Ichthyofaunal diversity and assemblages

2.1.1. Study area

Sundarbans, the largest delta in the world, consists of 10,200 km$^2$ of mangrove forest (Gopal and Chouhan, 2006), spread over India (4200 km$^2$ of reserved forest) and Bangladesh (6000 km$^2$ approx. of reserved forest) (Figure 2.1) and is also the largest mangrove forest in the world. Another 5400 km$^2$ of non-forest, inhabited region in India, along the north and north-western fringe of mangrove forest, is also known as Sundarban region in India. Hence, the total area of Sundarban region in India is 9630 km$^2$ which constitutes the Sundarban Biosphere Reserve between 21°40'04"N and 22°09'21"N latitude, and 88°01'56"E and 89°06'01"E longitude, is the smaller and western part of the complete Sundarban delta. It is part of the tide dominated lower deltaic plain. The landscape is characterized by a web of tidal water systems. The average tidal amplitude is between 3.5-5 meters, with the highest amplitudes in July-August and the lowest in December-January. The aquatic network of Sundarbans ecosystem of West Bengal is fabricated by several brackish water rivers and tidal canals. The mean depth of the estuary is 5 m and the maximum depth is 40 m near the mouth of the estuary. The river flow varies both seasonally and inter-annually, with a mean discharge of 400 m$^3$ s$^{-1}$ (Bettencourt and Ramos, 2003).
Ten study sites (Figure 2.2), with similar physical features (composed predominantly of well-packed soft mud) (Figure 2.3) were selected randomly along a 10 km stretch of Matla River, Bidya River, Boro Herobhanga rivulet and their adjacent mudflats in Indian Sunderban (22°01' N, 88°40' E). A small creek known as Boro Herobhanga rivulet (about 150 meter wide) joins two mighty river of Sundarbans namely the Matla River on the west and the Bidya River in the east. Matla is connected to Bidya and ultimately flows to the Bay of Bengal. The freshwater connection and discharge to this river has been lost in recent times. Salinity of the river water is relatively high owing to freshwater cutoff from upstream region (Manna et al., 2010). The sampling covered a total area of 35 km² with mean depth of 2.15±0.76 m and the tidal range of 3 m per 6 hr.
Figure 2.3: Pictures of the representative study sites (1-4) at high tidal and low tidal condition in the intertidal mudflats of Indian Sundarbans.
2.1.2. Habitat characterization and Environmental data

Prior to netting, important hydrological parameters viz. water current, water temperature, total dissolved solutes were measured using DYS handheld digital TDS/salt meter (Salt manager HDS 1024). Dissolved oxygen and pH were measured using Dissolved oxygen meter (Lutron DO 5509) and Hanna pH meter respectively. Turbidity was measured using Sechhi disc. Salinity (using Potassium dichromate as indicator and Silver nitrate as titrant), total acidity (using Phenolphthalein indicator, sodium thiosulfate and sodium hydroxide as titrant), total alkalinity (using methyl orange as indicator as sulfuric acid as titrant), total hardness (using ammonium buffer, Eriochrome black T indicator and EDTA sodium salt as titrant), inorganic phosphate (measured with persulphate digestion followed by spectrophotometry at 885 nm), nitrate nitrogen (measured with Cu-Cd column reduction and spectrophotometry), nitrite nitrogen (measured spectrophotometrically at 543 nm) and reactive silicate (measured with molybdate reagent and reducing reagent followed by spectrophotometry at 810 nm) were measured at high tidal environment following standard methods (Strickland and Parsons, 1972; Grasshoff et al., 1983; Grasshoff, 1983). Habitat characterization (Figure 2.4) was performed by measuring important habitat attributes (air...
temperature, herb density, shrub density, number of woody trees, canopy cover, litter cover, grass cover, root cover, canopy height, shrub height, shrub cover, distance from water, distance from forest type, litter depth, slope of terrain, aspects of slope, number of logs, girth of trees, number of trees with pneumatophore and number of burrows) (Heyer et al., 1994). The soil parameters (soil pH, soil moisture, soil organic matter, soil organic carbon, electrical conductivity, available phosphorus, available nitrogen and available potassium) were recorded during low tide in each sampling station following Brower et al. (1998).

2.1.3. Sampling

Sampling was conducted seasonally (pre-monsoon: February – May, monsoon: June – September and post-monsoon: October – January), between October, 2008 and February, 2012. Samples were collected fortnightly from each site during new and full moons. Fish sampling was performed during high tide with gill net of 20 m length with 3 cm spacing between adjacent knots, cast net of 1.2 m radius and with hook net of 50 m with 50 hooks, each at 1 m interval (Figure 2.5). The nets were
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placed at the onset of high tide and kept for approximately 6 h in order to ensure maximum fish catch per unit effort during high tide. 16 nettings were undertaken at each site of this estuary per season i.e. 4 netting per month at each site. Four nettings were pooled together and represented here as single abundance data for each site per month. The replicate sites were sampled simultaneously and for the same tenure to minimize the sampling errors (single abundance data from each site, i.e. 12 replicates per month, 48 replicates per season and 144 replicates per year). During low tide, few 100 m transects were established at each study site for studying the amphibious fishes. Along the transect 2 x 2 m quadrates constructed from nylon ropes and bamboo sticks were temporarily established at intervals of 8 m (approx. 10 quadrates per transect). Hand nets (dot net) and drag nets were also used for sampling during low tide.

The specimens were retrieved from the net, identified and species abundance was recorded to investigate species assemblages. The live fishes were measured for total length ($L_t$, cm) and weighed for total mass ($M$, g) and released after preserving individuals representing each fish species in 5% formalin. Fish specimens were identified to the lowest taxonomic level following existing literature (Shaw and Shebbeare, 1937; Day, 1958; Talwar and Jhingran, 1991).

The threat status of the fish species was obtained using the IUCN Red list Categories and Criteria version (IUCN, 1994). Species were categorized as: not evaluated (NE); lower risk-least concern (LR-LC), lower risk-near threatened (LR-NT).

2.1.4. Fish diversity, richness estimators, density and standing stock

To analyze the difference of alpha diversity between three seasons during inundated and exposed conditions, Species richness ($S$) (Margalef, 1957), Pielou's evenness ($J$) (Pielou, 1969), Species dominance ($D$) (Berger and Parker, 1970) and Shannon-
Wiener's (H') (Shannon and Weaver, 1949) diversity indices were calculated for high tide and low tidal conditions in three seasons.

To overcome sampling issues, non-parametric methods were also used to assess species richness. Several statistical estimators were used for calculating and extrapolating species richness; taking into account the possible proportion of rare species which reflected conservative estimates of the true species richness of an area (Colwell and Coddington, 1994). Jackknife method for species richness was applied to reduce the bias in case of estimation of species richness described in Heltshe and Forrester (1983). The expected and observed frequencies of correctly classified Jackknife results were compared by chi-square analysis. The bootstrap method (Smith and van Belle, 1984) and Chao’s estimator, Chao 1 requires species abundance data making species abundance curves (Chao, 1984). Comparisons of species richness across tidal scales (high tide and low tide) and seasonal scale (i.e. pre-monsoon, monsoon and post-monsoon) were carried out using the method of rarefaction – a statistical technique of estimating expected number of species for a given random sample of size n; species richness is then estimated as the sum of probabilities that each species will be included in the sample (Sander, 1968; Hurlbert, 1971). The computation was performed using Estimate S (version 8) software which uses Monte Carlo simulations of random samples drawn from the total set of samples for estimating the average species richness. Here, 200 randomizations were run for a given number of samples for the estimation of species richness values and their means were used in plotting the species accumulation curves.

Taxonomic diversity (species count per order) in three distinct seasons during high tide and low tidal condition was also studied as a means to reveal the diversity paradigm. For characterization of the biodiversity at the level of species diversity, rank abundance model was also constructed (King, 1964) (Table 2.1). Fish density and standing stock were calculated by dividing the total number and biomass of each species by the area sampled (35 km²).
Table 2.1: Elaboration of various diversity indices and estimators.

<table>
<thead>
<tr>
<th>Indices and Estimators</th>
<th>Equations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shannon-Weaver index (H')</td>
<td>( H' = -\sum p_i \ln p_i ) Where, the sum of ( p_i ) is the proportion of individuals found among these species.</td>
</tr>
<tr>
<td>Species evenness or equitability (J)</td>
<td>( J = H' / \log_2 S ) Where, ( H' = ) Shannon-Weaver index, ( S = ) observed number of species.</td>
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<tr>
<td>Species richness (S)</td>
<td>( S = (S_n - 1) / \log_2 N ) Where, ( S_n = ) observed number of species, ( N = ) total assemblage of species.</td>
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<tr>
<td>Dominance index (D)</td>
<td>( D = 1 / (n_{\text{max}} / N) ) Where, ( n_{\text{max}} = ) maximum abundance of species, ( N = ) total assemblage of species.</td>
</tr>
<tr>
<td>Species abundance curves (S_i)</td>
<td>( S_i = S_{\text{obs}} + (a^2/2b) ) Where, ( S_{\text{obs}} = ) observed number of species, ( a = ) number of species represented by 1 individual (singleton), ( b = ) number of species represented by only two individual (doubletons).</td>
</tr>
<tr>
<td>Chao 2 estimator (S_2)</td>
<td>( S_2 = S_{\text{obs}} + (L^2/2M) ) Where, ( S_{\text{obs}} = ) observed number of species, ( L = ) number of species that occur in one sample only, ( M = ) number of species that occur in exactly two samples.</td>
</tr>
<tr>
<td>Jackknife estimator (S_3)</td>
<td>( S_3 = S_{\text{obs}} + L (n-1/n) ) Where, ( L = ) number of species that occur in the sample only once, ( n = ) number of samples.</td>
</tr>
<tr>
<td>Bootstrap estimator (S_4)</td>
<td>( S_4 = S_{\text{obs}} + \sum_i (1-p_i)^n ) Where, ( p_i = ) proportion of samples containing species ( i ).</td>
</tr>
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</table>
Where, $E(S_n) = \text{expected number of species in a random}$

Rarefaction curves ($E(S_n)$) samples of $n$ individuals, $N = \text{total number of individuals}$, $S = \text{the number of species in the entire collection}$, $N_i = \text{the number of individuals of species } i$, $n = \text{the number of individual chosen for standardization}$. 

<table>
<thead>
<tr>
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<th>Equations</th>
</tr>
</thead>
<tbody>
<tr>
<td>$E(S_n) = \sum_{i=1}^{n} \left[ 1 - \left( \frac{N-N_i}{N} \right) \right]$</td>
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</table>

2.1.5. Ecological guild on the basis of migration

Species were classified by functional groups according to Franco et al. (2008) and each species was assigned to an ecological guild. The ecological guilds contained estuarine species (ES), marine migrants (MM), freshwater species (F), anadromous species (A) and catadromous species (C). Ecological guilds were analyzed both by number of species and number of individuals within each guild. The percentage contribution of each functional category to the total species richness and species abundance was calculated for two tidal conditions in each season and in each year, and compared to assess the prevailing function of each system during the time period of the present study.

2.1.6. Statistical analysis

Analysis of variance (ANOVA) was used to assess habitat differences in total fish density, standing stock, fish diversity indices and ecological guilds. Year and season were considered to be fixed factors. Fish density and standing stock data were transformed to log (x+1) in order to meet the assumption of homogeneity of variances (Underwood, 1997), whereas species richness, and diversity data were analyzed without any transformation, as the variances were homogeneous. As the study focused mostly on the temporal factors (seasons and years) in two tidal phases, one way ANOVA (Zar, 1999) was carried out separately with seasons (three levels: pre-
monsoon, monsoon, post-monsoon) and with years (year 1, year 2, year 3) as factors. Duncan test was used for post hoc comparisons after ANOVA. Statistical analyses were carried out by means of SPSS (Statistical Package for Social Sciences, version 17.0; Norušis, 2000).

Multivariate analyses were carried out on the fish density and standing stock data. These data were transformed by fourth-root and the Bray-Curtis similarity coefficient was calculated to generate similarity matrices (Clarke, 1993). For each season, an analogue of multivariate analysis of variance with a randomization test for significance (ANOSIM) was used to compare habitats in terms of their fish assemblage composition. Multidimensional scaling (MDS) was used to plot the relationships amongst habitats for each season where significant differences occurred. Similarity matrices used for MDS were also analyzed for the individual species making the greatest contribution to between-group differences (SIMPER; Clarke, 1993). Seasonal differences were investigated by performing ANOSIM for each habitat. All multivariate analyses were performed using PRIMER 5 (Clarke and Warwick, 1994) software.

Cluster analysis, using the Bray-Curtis similarity measure, was used to determine similarity between seasonal nekton assemblages for each year during high tide and low tide based on species abundance data, using PRIMER 5 software. Results were displayed using nonmetric multidimensional scaling plots, on which percentage similarity levels were assigned based on group-average linkage (Horinouchi, 2009). The abundance data were transformed square root when necessary. To determine whether or not major shifts in community structure have occurred between groups of seasons and between years, a non-parametric analysis, permutation-based two-way analysis of similarity (ANOSIM, Clarke, 1993) was performed. Where appropriate, R-statistic values for pair-wise comparisons provide by ANOSIM was used to determine the dissimilarity between groups. Values close to 1 indicate very different composition, while values near zero show little difference. It was used to test the null
hypothesis that within seasons no changes in community structure were observed between years, and to test that within year, no changes in community structure were observed between seasons separately for high and low tidal conditions.

Canonical Correspondence Analysis (CCA) (Ter Braak, 1988; McGarigal et al., 2000) was performed in order to elucidate the possible relationships between biological assemblages of species and the hydrological variables during two tidal phases. The seasonal samplings from all the study sites in three years were included in the analysis. Rare species in the fish species matrix were down weighted following the CANOCO procedure in order to prevent their having an excessive influence on the ordination. This analysis was carried out using CANOCO (version 4.5) software.

2.2. Resource partitioning and trophic guild analysis

2.2.1. Stomach content verification

Stomach contents analysis is primarily a method for qualitative estimation of dietary composition by investigation of prey items in the fish stomach. This type of analysis is often used in field studies, and summarize of the methods are given by Hynes (1950), Windell and Bowen (1978), Hyslop (1980), Bowen (1996) and Chipps and Garvey (2002).

The fishes were anaesthetized with MS222 (>15 specimens per species) and each stomach was visually assessed for fullness (1=empty, 2=25%, 3=50%, 4=75%, 5=100% full), and those with a score of 3 to 5 were dissected. Various taxa digest at different rates, as such, all recently consumed taxa may be present in the foregut but only resistant items remain in the hindgut. To avoid bias when both easily digested prey and resistant prey are present, only the immediate foregut (e.g., stomach) were sampled in the fishes where prominent stomach is absent. The contents of the stomach or foregut were collected separately in 70% ethanol and observed under
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microscope. Prey items were identified to the lowest possible taxon and each individual item was counted.

2.2.2. Stomach content analysis

A frequency of occurrence (%F) method was employed to analyze stomach contents for the determination of diet composition as described by Hyslop (1980). Prey-specific abundance is a function of the percentage of prey items in only those species in which the prey occurs (Amundsen et al., 1996).

2.2.3. Feeding guild formation

A cluster analysis, using the Bray-Curtis coefficient measure was performed in order to group species based on diet similarity using PRIMER 5 software. A dendrogram was constructed for hierarchical cluster analysis to describe the similarity in feeding habitat among the teleosts based on the %F values (Cabral and Murta, 2001).

2.2.4. Niche breadth and Niche overlap

To determine diet specialization of each species, dietary breadth was calculated based on %N for each prey according to Levin's standardized index (Hurlbert, 1978; Krebs, 1989). This index ranges from 0 to 1.0; where values close to 0 indicate specialization while values close to 1.0 show generalization (Gibson and Ezzi, 1987).

To calculate dietary overlap, %N was applied to the index proposed by Pianka (1980) which is a symmetric analysis that allows approximations to overlap between two species in one way; values = 0.6 are considered “biologically significant” for teleosts (Pianka, 1976). To validate significance of these overlaps, the observed values were compared to a distribution of expected overlap values based on null-model simulations. The distribution of null model data resulted from 1000 randomizations of the diet by using EcoSim (version 7) software (Gotelli and Entsminger, 2001). The observed value was considered statistically different from the null distribution if it was greater or less than the simulated index 95% of the time (p<0.05; Winemiller and
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An observed value significantly less than the simulation index would suggest differences in diet or diet partitioning while an observed value significantly higher than the simulation index would suggest similarities in diet or the lack of competition for food resources (Winemiller and Pianka, 1990).

2.2.5. Trophic diversity

Trophic diversity (D) (Herrera, 1976), was analyzed by sampling 100 L of the water using conical type circular tow net of 35 μm mesh size from the habitat during high tide and submerged soil samples were analyzed during low tide to collect all available prey types. The contents were also preserved in 4% formaldehyde solution and analyzed in laboratory. This was practices in 12 replicates. Zooplanktons and other aquatic organisms were identified following Hutchinson (1961), Arora and Kosin (1966) and Dhanapati (1974).

Similarly trophic diversity (D) was also measured in the stomach or foregut of fishes. Rarefaction curves (Hurlbert, 1971) were also used to see the diversity of prey populations predated by fishes, assembled during high and low tidal mudflats. The total number of food items consumed by each stage gives the richness of the prey consumed.

2.2.6. Prey preference

Chesson's α index provides a measure of the proportional abundance of a prey type in fish diet relative to its proportional abundance in the habitat (mudflat) (Chesson, 1983). Chesson's index is commonly used in fish diet research, and is considered one of the most reliable for field-based observations (Lechowicz, 1982). Chesson's α ranges between 0 (strong negative selection) and 1 (strong positive selection), with a value of 0.06 indicating non-selective feeding for five prey classes. An overall Chesson's α value was calculated by pooling mean α values across dates; variance for the pooled α value was estimated using the method of Manly (1974). Overall α values...
for each species were tested against the neutral selectivity value using Student's t-test (Manly, 1974). Chesson’s α values met the assumption of normality following arcsine, square root transformation. Individual t-tests for each plankton species were assessed at an adjusted probability level of 0.01 (0.05/5) (Table 2.2).

<table>
<thead>
<tr>
<th>Indices</th>
<th>Equations</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Frequency of occurrence (%Fi)</strong></td>
<td>( %F_i = \left( \frac{N_i}{N} \right) \times 100 )</td>
</tr>
<tr>
<td>Where, ( F_i ) = percent frequency of prey type i, ( N_i ) = number of shad with prey i in the stomach, and ( N ) is total number of shad with stomach contents.</td>
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<tr>
<td><strong>Prey specific abundance (p)</strong></td>
<td>( p_i = \left( \frac{\sum S_1 / \sum S_{t_i}}{100} \right) )</td>
</tr>
<tr>
<td>Where, ( S_i ) = the stomach content comprised of prey i, ( S_{t_i} ) the total stomach content in only those individuals with prey i in their stomach.</td>
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</tr>
<tr>
<td><strong>Index of Relative Importance (IRI)</strong></td>
<td>( IRI = F % \left( N % + W % \right) )</td>
</tr>
<tr>
<td>Where, ( F ) = frequency of occurrence of a food item in stomach, ( N ) = numerical percentage of food item in stomach, ( V ) = percentage by volume of the prey item in stomach.</td>
<td></td>
</tr>
<tr>
<td>Modified IRI: ( %IRI = \left( \frac{IRI}{\sum IRI} \right) \times 100 )</td>
<td></td>
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<tr>
<td><strong>Diet breadth (Levin’s standardized index) (B)</strong></td>
<td>( B_i = \left( \frac{1}{n - 1} \right) \left( \frac{1}{\sum p_{ij}^2 - 1} \right) )</td>
</tr>
<tr>
<td>Where, ( B ) = Levin’s standardized index for predator i; ( p ) = proportion of diet of predator i that is made up of prey j; and ( n ) = number of prey categories.</td>
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<tr>
<td><strong>Pianka’s niche overlap index</strong></td>
<td>( O_{jk} = \sum_{i=1}^{n} \left( P_{ij} \times P_{ik} \right) / \sqrt{\sum_{i=1}^{n} P_{ij}^2 \sum_{i=1}^{n} P_{ik}^2} )</td>
</tr>
<tr>
<td>Where, ( O_{jk} ) = niche overlap index between predator species j and species k, ( P_{ij} ) = proportion of prey species i in relation to the total amount of prey ingested by species j. ( P_{ia} ) = proportion of prey species I in relation to the total amount of prey ingested by species k, ( n ) = total no. of prey species.</td>
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</table>
Rarefaction curves

Where, $E(S) = \text{expected richness in the rarefacted sample with a given } n$, $n = \text{standard size of the sample}$, $N = \text{total number of quotations of each kind of food}$, and $N_i = \text{number of meals with the } i^{th} \text{ food item}$. The computation was performed using EstimateS software.

\[
E(S_n) = \sum_{i=1}^{n} \left[ 1 - \frac{(N-N_i)}{\binom{N}{n}} \right]
\]

Chesson’s $\alpha$ value

Where, $r_i = \text{relative abundance of prey species } i \text{ in the stomach}$, $p_i = \text{relative abundance of prey species on the mudflat}$, $m = \text{no. of prey species}$.

\[
\alpha = \left( \frac{r_i}{p_i} \right) / \sum_{i=1}^{m} \frac{r_i}{p_i}
\]

2.3. Digestive enzyme analysis

Proper utilization of dietary nutrients i.e. conversion into body components depends on the digestibility of the natural food stuff, which is the function of different chemical processes that helps to hydrolyze and breakdown components. After collection of the stomach content, liver, stomach and intestine of thirty one species of fishes previously anaesthetized, were dissected out, weighed, kept in liquid nitrogen during transportation to the laboratory and frozen at -70°C until assay of the enzymes.

2.3.1. Tissue extracts preparation

The preparation of tissue extracts was carried out at 4°C. The digestive organs of each fish (liver, stomach and intestine) were thoroughly washed with chilled glass-distilled water and homogenized in 0.02 M phosphate buffer pH 7.0 (1:5 w/v) for 3 min at 5500g, 4°C. Tissue homogenates were centrifuged in a Hermule Z323K refrigerated centrifuge at 10,000g for 25 min at 4°C. The supernatant was separated.
and preserved for enzyme assays. Five digestive enzymes were assayed at the optimum temperature in all the samples.

2.3.2. Determination of total protein

The soluble protein content of each extract was determined against bovine serum albumin (BSA) as reference (Lowry et al., 1951). Aliquots of 0.05 ml of the tissue homogenates were taken in test tubes and 0.45 ml double distilled water were mixed, with it 5 ml of reagent C (2% sodium carbonate in 0.1 N sodium hydroxide (reagent A) and 0.5% CuSO₄, 5 H₂O in 0.1% sodium potassium tartrate (reagent B) mixed in 50 ml of reagent A to 1 ml of reagent B) were added and incubated for 30 min at 37°C, 0.5 ml of 1N Folin reagent were added to these mixtures and re-incubated for 10 min. Optical density were measured at 660 nm against inactivated enzyme solution-reagent blanks. Protein calibration graphs were plotted from absorbance of serial dilutions of BSA stock solution in distilled water.

2.3.3. α-Amylase activity

α-Amylase activity was assayed as modified method of Bernfeld (1955), using starch (1%) [Sigma, U.S.A.] as substrate and double phosphate (Na₂HPO₄ + NaH₂PO₄) buffer (pH 6.9). The test solutions were read against same amount of inactive enzyme, buffer and substrate blank solutions. Maltose, released by hydrolysis of substrate, was measured by reaction with dinitro salicylic acid (DNSA). Activity was determined from a standard curve of D (+) maltose at 540 nm absorbance. One unit (U) of α-amylase was defined as the amount of enzyme needed to hydrolyze 1 mg of starch per min at 37°C. The α-amylase activity was expressed per mg of protein.

2.3.4. Cellulase activity

Cellulase activity was determined following Kesler and Tulou (1980), using carboxy-methyl-cellulose (1%) [Sigma, U.S.A] as substrate and double phosphate buffer (pH 5.5). The hydrolyzed products were measured by reaction with DNSA in 1:1
proportion at 540 nm. Activity was compared from a standard curve of D (+) glucose absorbance. One unit of cellulase was defined as the amount of enzyme needed to hydrolyze 1 mg 1% CMC per min at 37°C.

2.3.5. Invertase activity

The invertase activity was estimated following Pal et al. (1980) using (2.5%) sucrose [Sigma, U.S.A.] as substrate, 0.2N NaCl for activation and double phosphate buffer (pH 5.5). Hydrolyzed products were measured with DNSA in 1:1 proportion. Activity was determined from a standard curve of D (+) glucose absorbance at 540 nm (Bacon, 1955). A unit of invertase was defined as the amount of the enzyme needed to hydrolyze 1 mg of substrate per min at 37°C.

2.3.6. Alkaline protease activity

Alkaline protease was measured following Ichishima (1970) using (1%) bovine serum albumin [Sigma, U.S.A] as substrate (pH 10.0). 5% TCA was used for halting the reaction. Supernatant of this solution were measured in 4% Na2CO3 solution and double diluted Folin solution at 620 nm. One unit of alkaline protease activity was calculated as the amount of enzyme needed to hydrolyze 1 mg BSA per min at 37°C.

2.3.7. Pepsin activity

Pepsin was measured following Ragyanszky (1980) using casein (1%) [Sigma, U.S.A.] as substrate at pH 1.5 using 60 mM HCl. For alkaline proteases as well as for pepsin, tyrosine was used as standard. Enzyme assays were performed with a Shimadzu UV-1700 PharmaSpec, UV/visible spectrophotometer at 720 nm. Activity of all the enzymes was expressed in units per mg of protein (U mg⁻¹ protein).

2.3.8. Statistical analysis

Multivariate Analysis of Variance (MANOVA) (Zar, 1999) was applied using SPSS 17.0. The mean value of fifteen repetitions of each enzyme from each tissue evaluated
for each fish species was used to interpret the variations among the species. The homogeneity between mean values of the different fish species was tested using post hoc Duncan test; values were considered statistically different at the \( p<0.05 \) level.

Dendrogram was constructed for hierarchical cluster analysis among the teleosts for digestive enzymes using Ward method and Euclidean distance (SPSS 17.0).