

## **Ecological quality status assessment of tropical estuaries with benthic indices using differently derived reference conditions**

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### **Abstract**

Setting appropriate reference conditions (RCs) is critical for classifying the Ecological Quality Status (EcoQS) which is extremely challenging, considering the present-day estuaries. The EcoQS of three tropical estuaries was assessed by applying five different RCs to identify the best applicable method for the area. The AZTI's indices (AMBI and M-AMBI) categorised areas with sharper pollution gradient with ease, while classification of moderate-low polluted areas was ambiguous. Indices responded to chemical stressors more clearly in the impacted estuary compared to the less polluted estuaries. Ecological status assigned by the default RC (lowest AMBI and highest diversity and richness values) were more accurate than those obtained on application of other four types of RCs, owing to various estuarine constraints that are discussed herewith. Thus, prior to application, caution should be exercised while setting area-specific RCs. The inclusion of combination of AZTI's indices with professional judgement for successful appraisal of ecosystem is recommended.

**Keywords:** Macroinvertebrates; benthic indices; reference conditions; ecological quality status; salinity zones; tropical estuaries .

## 1. Introduction

Estuarine ecosystems, being the fulcrum of urbanisation and industrialisation, are degrading at an accelerated rate due to the multitude of anthropogenic stressors imposed on it which eventually affect the resident biota (Hess et al. 2020). The consistent deterioration has prompted periodical assessments of coastal and estuarine ecological health to evaluate their status and apply corrective interventional actions to improve ecological health as deemed necessary. Towards this, over the past decades, various methods have been developed which include simple univariate metrics and multivariate analyses that combines the biological components together with physico-chemical and environmental pollutants (Borja et al. 2012, Berthelsen et al. 2018). The benthic realm serves as an important indicator of ecological health status and macroinvertebrates are regarded as the backbone of monitoring programmes in many studies due to their varying sensitivities to various pollutants (van der Linden et al. 2016, Qiu et al. 2018, Moreira et al. 2019, Yan et al. 2020). Benthic Biotic indices (BBIs), which were constructed based on the sensitivity of organisms to environmental stressors, have flourished as effective tools in describing the ecological health status (Borja et al. 2015, Yan et al. 2020). Among the surfeit of benthic indices available, AZTI's Marine Biotic Index (AMBI) (Borja et al. 2000) and multimetric AMBI (M-AMBI) (Muxika et al. 2007) are extensively used worldwide due to their sensitivity to various pollutants and applicability in different coastal segments and geographical areas (Qiu et al. 2018, Borja et al. 2019, Santibañez-Aguascalientes et al. 2020). Although the index application is user friendly, it includes a complex process in which the definition of different typologies and biological reference conditions are required (Pardo et al. 2020). Reference conditions represent undisturbed conditions or a pristine set up with absence or minimal pressure (Muxika et al. 2007). To assess the ecological quality status (EcoQS), Borja et al. (2012) have mandated three steps: (i) definition of a reference condition, i.e. the conditions of the indicator in pristine sites or in areas without any anthropic stress; (ii) computation of the chosen indicator against these reference conditions and the calculation of an Ecological Quality Ratio (EQR); and (iii) use of a conversion scale for transforming the EQR into an EcoQS. An exhaustive review of 83 studies found that a major shortcoming for the failure of M-AMBI was the setting of inadequate reference conditions (Borja et al. 2019). Thus, the Reference Condition (RC) identified dictates the accuracy of the ecological status conferred by the benthic index (Borja et al. 2012).

The complexity of distinguishing natural and anthropogenic stress in estuaries is described as the 'Estuarine Quality Paradox' (Dauvin and Ruellet, 2009). This paradox, along with the absence of

historical data and undisturbed or pristine areas make it difficult to define reference conditions in estuaries while classifying the EcoQS (Hess et al. 2020). Additionally, identifying reference conditions in estuaries is more cumbersome, given the high natural variability of these dynamic systems resulting in a mosaic of habitats. Salinity is known to be the most important factor in estuaries driving the spatial distribution of macrobenthic taxa along the gradient (Chainho et al. 2007, Borja et al. 2012, Van Diggelen and Montagna, 2016). Therefore, the conditions prevailing in the upstream differ from the downstream in numerous ways which again pose difficulties in arriving at appropriate reference conditions (Santos et al. 2021). Hence, type-specific reference conditions are usually derived for each salinity zone while assessing the EcoQS, since each habitat comprises of species with specific characteristics (Bald et al. 2005, Basset et al. 2013).

Various approaches have been suggested for setting of reference conditions in marine and transitional waters (Borja et al. 2012, Borja et al. 2019, Hess et al. 2020, Santibañez-Aguascalientes et al. 2020). Different methodologies adopted so far for deriving reference conditions are: (i) data acquired from a pristine or undisturbed site; (ii) historical information or background data; (iii) quantitative models or (iv) best professional judgement (Bald et al. 2005, Borja et al. 2004, Borja and Tunberg, 2011, Forchino et al. 2011). These approaches along with some new methodologies have been reviewed by Borja et al. (2019) wherein the merits and demerits of each have been extensively discussed.

The tropical estuaries in the Indian subcontinent are suffering from unprecedented stress due to exposure to various anthropogenic activities (industrial development, sewage disposals, etc) (Dias et al. 2018, Mulik et al. 2020a). This makes it challenging to derive reference conditions because of unavailability of sites with minimal impact, paucity of historical data and absence of numerical models (Feebarani et al. 2016). The AZTI's indices have been the most frequently and successfully applied BBI for establishing the ecological status of the Indian estuaries and coastal waters (Feebarani et al. 2016, Mulik et al. 2017, Dias et al. 2018, Mulik et al. 2020b). Most of these studies have followed a set approach to establish reference conditions i.e. by increasing the highest richness and diversity values to 15% for each zone as suggested by Forchino et al. (2011) which was considered reasonable by many authors (Borja and Tunberg, 2011, Paganelli et al. 2011, Cai et al. 2014, Wang et al. 2020). In a scenario where other methods of deriving reference conditions are reported, it is of interest to investigate the extent of influence of the choice of reference conditions eventually has on the quality of ecological status derived.

In the present investigation, three estuaries were chosen, namely Kundalika, Vashishti and Shastri which have been exposed to differential perturbations. The Kundalika was previously assessed for EcoQS to test the efficiency of different benthic indices along the estuarine gradient by Dias et al. (2018). The authors reported satisfactory results along the zones using M-AMBI by means of the above-mentioned approach for reference conditions which gave a final EcoQS. Hence, the present study will draw attention to the effectiveness of the well-suited index in the Kundalika by comparing the results with the past data and thus, showing its consistency in assessment studies. It will also provide information on the current EcoQS of the Kundalika in order to check if the estuary has improved or deteriorated overtime. It is crucial to enhance the existing knowledge and to set a baseline for other estuaries (Vashishti and Shastri), since there is no available database along the northwest coast of India. However, estuaries can change over time depicting different water types depending on their intrinsic characteristics and may vary in terms of perturbation levels. Therefore, we emphasise that there is a pressing need to clearly define the adequate reference conditions for the estuaries as the reliability in the EcoQS interpretation depends on these conditions (Borja et al. 2012). Against this backdrop, the study aimed (i) to assess the EcoQS of three estuaries along the salinity gradient by employing AZTI's indices, (ii) to identify the suitable reference conditions for the index application that can aid accuracy of EcoQS assignment, and (iii) to highlight the strengths and challenges of deriving reference conditions for these estuaries.

## **2 Materials and methods**

### **2.1 Study sites**

In the present investigation, three estuaries were chosen along the Northwest coast of India: Kundalika (18°31'21.72" N 72°56'01.40" E), Vashishti (17°34'06.95" N 73°13'40.11" E) and Shastri (17° 18' 54.1" N 73° 8' 13.2" E) (Fig. 1). Kundalika is a shallow 65 km long estuary (825 km<sup>2</sup>) originating in the Sahyadris near the Garbolot Pass which joins the Arabian Sea after a westerly course. The Vashishti covering a length of 50 km with an area of 2238 km<sup>2</sup> originates in the slopes of the Western Ghats and meets the Arabian Sea at main town of Dabhol. Tides are of semi-diurnal nature in both the areas. The Shastri having a length of 29 km with area coverage of 2174 km<sup>2</sup> emerges from Prachitgad historical fort in the Sahyadri Forest Reserve at an elevation of 839 m above sea level flowing down in the west of Sahyadris before meeting the Arabian Sea near Jaigarh town. This estuary experiences limited tidal impact and tidal ingress is mostly in the middle and upper segments. Shastri does not receive any known point discharges of waste water and harbours luxuriant mangroves (NIO 2017). With rapid industrialisation and urbanisation lining the mid-upper

regions of both Kundalika and Vashishti estuaries, they are exposed to considerable amounts of anthropogenic pressures. Industrial effluents are released into these estuaries after minimal treatment (NIO 2009, NIO 2018). Additionally, these study areas receive domestic wastes of about 1.4 MLD in Kundalika and 7.7 MLD in Vashishti (NIO 2009). In the downstream region of Kundalika, a fishing port is situated which is an important fish landing centre for the district (NIO 2018). The estuary is subject to dredging activities and barge movements (Dias et al. 2018). The lower zone of Vashishti estuary is shallow generally >2m during spring tides due to presence of sand bars which reduces the navigational advantage of the river (NIO 2018). This estuary is subjected to sand mining and also receives agricultural wastes (Dandekar 2010, Nasnodkar and Nayak, 2018).

## **2.2 Sampling design and laboratory analyses**

Two field surveys were conducted in the estuaries during November to January 2016 (postmonsoon) and March to April 2016 (premonsoon) with a grid of 10 sampling sites in Kundalika (K1-K10), Vashishti (V1-V10) and 6 stations in Shastri (S1-S6). Bottom water samples were collected in duplicates at each station using Niskin sampler for the determination of physico-chemical parameters where the dissolved oxygen (DO) was fixed immediately with Winklers A and B (Grasshoff et al. 1999). Nutrient samples ( $\text{NO}_3^-$ -N,  $\text{NO}_2^-$ -N,  $\text{NH}_4^+$ -N and  $\text{PO}_4^{3-}$ -P) were kept at -4°C and transported to the laboratory for further analyses. Sediment samples were collected with the help of a van Veen grab (0.04m<sup>2</sup>) for the grain size texture analysis and total organic carbon (TOC) content which were placed in high density tarsons bottles and kept in ice. For the macrobenthic samples, the sediment was collected using van Veen grab in quadruplicates; where in total 208 samples were obtained (Kundalika and Vashishti = 4 replicates x 20 stations x 2 seasons; Shastri = 4 replicates x 6 stations x 2 seasons). The sediments were washed, sieved using a sieve of 0.5mm, stained with Rose bengal stain and fixed with 5% formaldehyde.

In the laboratory, the water samples were analysed to estimate the salinity using an ‘Autosal’ laboratory salinometer (model: 8400B, Guideline Instruments Ltd.). The fixed DO samples and nutrient samples were estimated using the protocol by Grasshoff et al. (1999). The sediment texture for sand, silt and clay compositions was analysed using the wet-sieving pipette method (Buchanan et al. 1984). The sediments for TOC were oven-dried at 85°C, homogenised using agate mortar and pestle followed by estimation using the TOC analyser of SHIMADZU SSM-5000A series make. The macrobenthic samples were further sorted, counted and identified up to the lowest possible taxonomic level using stereo and compound microscopes by referring the available identification keys Day 1967; Brinkhurst 1971; Fauchald 1977; Lincoln 1979; Lyla et al. 1998; Chapman 2007.

The species names were confirmed using the World Register of Marine Species (WORMS) webpage available from <http://www.marinespecies.org> and data for abundance was enumerated as ind.m<sup>-2</sup>.

## 2.3 Calculation of indices

The detailed schematic representation of steps involved in the calculation of EcoQs is given in Fig. 2.

### 2.3.1 Univariate indices

The macrobenthic species abundance data were subjected to univariate metrics to estimate the total density (N); number of species (S); species richness,  $d$  (Margalef Index); species evenness,  $J'$  (Pielou's), Shannon-Wiener Index,  $H'(\log 2)$ , Simpson's index ( $1-\lambda'$ ) and Hurlbert index ( $ESn$ ). The macrobenthic species obtained were divided into six taxonomic levels (species, genus, family, order, class and phylum) to calculate the taxonomic diversity ( $\Delta$ ) and taxonomic distances of individual species including the average taxonomic distinctness ( $\Delta^*$ ), the variation in taxonomic distinctness ( $\Delta^+$ ) and the total taxonomic distinctness ( $\Lambda^+$ ). These provide a measure of the average spread of species across higher taxa, where  $\Lambda^+$  is a measure of the evenness of the spread of species across higher taxa (Warwick and Clarke, 1995, Clarke and Warwick, 2001). All univariate analyses were carried out using the software package PRIMER v.7 (Clarke and Gorley, 2015).

### 2.3.2 Benthic indices: AMBI and M-AMBI

The macrobenthic species were assigned to one of the five ecological groups (EG) according to their sensitivity or tolerance level such as EGI (sensitive species), EGII (indifferent species), EGIII (tolerant species), EGIV (second-order opportunist species) and EGV (first-order opportunist species). The species were assigned according to the list of December 2020 which is accessible freely on the AMBI software (<http://ambi.azti.es>). For the species not available on the list, EG of the same genus was assigned (Borja and Tunberg, 2011). Further, the AMBI was calculated for each zone separately using the replicate data to assess the EcoQS of the three estuaries.

The multivariate index is an extension of AMBI based on factorial analysis of AMBI, species richness (S) and Shannon–Wiener diversity ( $H'$ ) values. As M-AMBI depends on the reference conditions (RC), the derivation of type-specific RCs is a mandatory step especially in estuaries (Basset et al. 2013). As suggested by the European Commission, 2003, the reference conditions should represent the 'high' ecological status for areas with total absence of anthropogenic disturbance and the 'bad' ecological status for those with high impact of disturbances. At 'high' status, the reference condition may be regarded as optimum where the M-AMBI approaches one and

at 'bad' status, the M-AMBI approaches zero. In particular, the reference values of the three metrics (AMBI, S and H') are required for the calculation of the M-AMBI (Paganelli et al. 2011). Generally, when calculating the M-AMBI in the AMBI software, the output is generated based on the default RCs that is lowest AMBI value and highest S and H' values which are derived from the macrobenthic dataset in the targeted area. However, this could not produce accurate results in many studies and the need for modification of RCs was highlighted. We attempted to calculate the M-AMBI by testing the different types of RCs in order to assess the accurate EcoQS.

Five types of RCs were employed to assess the efficacy of M-AMBI. 1. The default M-AMBI RC (RCI), 2. Increasing the S and H' values by 15% for the whole dataset of each estuary (RCII) (Cai et al. 2014), 3. Here, after determining the salinity stretches, we set default RCs zone-wise for each estuary (RCIII), 4. Increasing the S and H' values by 15% separately for the zone-wise data (RCIV), 5. The 'Virtual Reference Conditions' as proposed by Borja et al. (2004) in which only the sensitive group of species (EGI, EGII & EGIII) are considered and the opportunists (EGIV & EGV) are removed from the analysis (RCV). This methodology was adopted due to the absence of pristine sites and lack of historical data (Hutton et al. 2015). We used this dataset to calculate AMBI, S and H' and we calculated the 95<sup>th</sup> percentile of S and H' values and 5<sup>th</sup> percentile of AMBI to set 'High' RCs for each zone. For the 'Bad' RCs, we used the azoic conditions (AMBI=6, S=0 and H'=0) as described by Muxika et al. (2007). Further, M-AMBI was calculated using the different types of RCS (Table 1). The threshold boundaries used for the AMBI classification are based on those proposed by Borja et al. (2000, 2007): 'High', 0.0-1.2; 'Good', 1.2-3.3; 'Moderate', 3.3-5.0; 'Poor', 5.0-6.0; and 'Bad', 6.0-7.0. The threshold values for the M-AMBI classification (Muxika et al. 2007) are: 'High' quality, >0.77; 'Good', 0.53-0.77; 'Moderate', 0.38-0.53; 'Poor', 0.20-0.38; and 'Bad', <0.20.

## **2.4 Statistical treatment**

Canonical Correspondence Analysis (CCA) was carried out to analyse the influence of environmental variables on the distribution of macrobenthic species based on preliminary Detrended Correspondence Analysis (DCA) (Hill and Gauch, 1980). The matrix was constructed for each estuary based on the 11 environmental variables and species having >2% of relative abundance in order to obtain better visualization on the CCA plots using the CANOCO version 4.5 (ter Braak and Smilauer, 2002). To test the significance of the ordination axes, Monte Carlo Permutation test (499 unrestricted permutations) was considered. Spearman's rank correlation coefficients were used to visualize significant relationships between univariate indices, AMBI, M-AMBI based on different RCs and abiotic variables using the STATISTICA 7 software package.

### 3. Results

#### 3.1 Characterisation of estuarine zones

The sampling stations of the estuaries were classified into three main zones based on the salinity ranges: euhaline (>30), poly-mesohaline (5-30) and oligohaline (0-5) (Bald et al. 2005). All three zones were exhibited in Kundalika and Vashishti, whereas Shastri had only the euhaline zone due to fewer sampled stations restricted only to the lower part of estuary (Table 2). As this is a crucial step for setting of reference conditions, all our results are based on the above-mentioned salinity zones.

#### 3.1 Physico-chemical variables

As represented in Table 2, the hydro-sedimentological variables measured in all the estuaries gave different results depending on the nature of the estuary. The average DO levels were relatively lower in the poly-mesohaline zones of the estuaries (101.6-136.6  $\mu\text{mol l}^{-1}$ ), while the euhaline zones of all the estuaries had the highest values in the range of 183.3-193.0  $\mu\text{mol l}^{-1}$ . The average nutrient concentrations indicated elevated values in the poly-mesohaline zones of Kundalika and Vashishti whereas lower concentrations were measured in Shastri (Table 2). Higher SS concentrations were recorded in the Kundalika (143.8- 180.5  $\text{mg l}^{-1}$ ) relative to other estuaries. The silt fraction dominated the euhaline zones (>50%) of all the three estuaries. The sand composition was higher in the other two zones of Vashishti and the oligohaline zone of Kundalika. The average  $C_{\text{org}}$  content was highest in the oligohaline zone of Vashishti ( $3.5\pm 0.4\%$ ) and poly-mesohaline zone of Kundalika ( $2.8\pm 0.8\%$ ), where as in the other estuarine zones the values were <2.0%.

#### 3.3 Macrobenthic species assemblages

The species density varied spatially depending on the zone in each estuary (Table 4). In Kundalika, a total of 82 taxa (13244 individuals) were identified, the dominant taxa being the polychaete, *Namalycastis indica* (14.8%), oligochaete, *Tubificoides pseudogaster* (9.3%) and polychaetes, *Prionospio cirrifera* (8.8%), *Paraprionospio patiens* (8.6%) and *Neanthes glandicineta* (6.0%). Vashishti samples yielded a total of 85 taxa (34138 individuals) which was dominated by the amphipod, *Corophium volutator* (34.2%), bivalve, *Irus* sp. (9.0%), and amphipods, *Eriopisella sechellensis* (8.4%) and *Gammaropsis* sp. (7.2%). The Shastri was characterised by 82 taxa in total (32913 individuals), which was dominated by the bivalve, *Astarte montagui* (21.2%) and polychaetes such as Maldanidae (11.7%), *Paraprionospio patiens* (9.7%) and *Cossura coasta* (6.9%). The macrobenthic abundance was higher in the oligohaline and lower in the euhaline zones of both



Kundalika and Vashishti (Table 3). The species diversity measures displayed highest values in the euhaline zones, which subsequently decreased towards the oligohaline zones.

### **3.4 Benthic indices**

#### **3.4.1 EcoQS based on AMBI**

The macrobenthic dataset consisted of 145 taxa in total considering all the estuaries, of which 141 were ascribed to an ecological group (EG) according to the AZTI species library and four were not assigned due to broad taxonomic resolution (Table 4). The mean EG percentages varied across the zones of each estuary (Fig. 3). The Kundalika indicated the highest presence of EGIV species in all the zones. In Vashishti, the euhaline zone was dominated by the EGI (33%) and EGII (29%) species, poly-mesohaline zone was dominated by EGIII (52%) species and the oligohaline was chiefly inhabited by EGI (84%) species. The euhaline zone of Shastri was occupied with the EGI (53%) followed by EGIV (22.5%) species. The mean AMBI values (Fig. 4) in all estuarine zones varied between 1.1 and 3.0 indicating a slightly disturbed class (good), the exception being the oligohaline zone of Kundalika (5.4; poor EcoQS). However, the stations occurring at each zone showed different EcoQS (Table A.1) depending on the species composition and their sensitivities that resulted in variable values for EcoQS assessment.

#### **3.4.2 M-AMBI application using different reference conditions**

Different scenarios were defined based on the five reference conditions (Table 1) employed for the application of M-AMBI in each estuary (Fig. 4). In Kundalika, the euhaline zone was assigned a 'good' EcoQS similarly by all the RCs. The EcoQS varied widely in the other two zones switching between 'high' and 'bad' status indicating the highest zone-to-zone variability. The variability was limited in the other two estuaries for specific zones and different RCs wherein the EcoQS fluctuated occasionally between the adjacent categories of 'good' and 'high'. It was also observed that RCI, RCII and RCV produced same results, whereas RCIII and RCIV assigned similar EcoQS to the different segments of Kundalika. Based on RCI and RCII, a 'good' status was assigned to all the zones of Vashishti. The oligohaline zone was assigned as 'high' and the other two zones as 'good' status by RCIII, RCIV and RCV. The Shastri had only one zone (euhaline) and consequently only three RCs were applicable which gave 'good' (RCI and RCII) and 'high' EcoQS (RCV) status. The station-wise M-AMBI produced different EcoQS (Table A.1) in accordance with the species sensitivities. Overall, the aggregated/mean EcoQS at each zone indicated that the ecological status of Kundalika was inferior to the other two estuaries. However, comparison of the present study (2015-

2016) with the previous study (2014) of Kundalika indicated that the estuary had improved in the intervening period (Fig. A1).

### **3.5 Link between the indices and environmental variables**

The Spearman correlation between indices and environmental variables revealed numerous significant correlations (Table 5). In Kundalika, AMBI was negatively correlated with salinity but positively correlated with  $\text{NH}_4^+\text{-N}$ . The M-AMBI based on different RCs (RCI, RCII & RCV) showed strong positive correlations with salinity and negative correlations with nutrients. However, the RCIV was negatively correlated with DO. Similarly, all other univariate indices correlated positively with salinity and negatively with nutrients. In Vashishti, a significant positive correlation was observed between AMBI and salinity. None of the M-AMBI's reference conditions showed any correlations except for RCII which was negatively correlated with  $\text{PO}_4^{3-}\text{-P}$  in this estuary. Few univariate measures were correlated with sediment grain size and other abiotic parameters. In Shastri, only AMBI was positively correlated with  $\text{C}_{\text{org}}$ .

CCA was performed on the macrobenthic species and environmental variables in each estuary (Fig. 5). Stations were primarily clustered along the salinity gradient in Kundalika and Vashishti. The euhaline zone in Kundalika was marked by high salinity, DO and silt content; poly-mesohaline zone was associated with high nutrients,  $\text{C}_{\text{org}}$  and clay, while the oligohaline zone had higher composition of sand. In Vashishti, the euhaline stations were associated with high salinities, DO and  $\text{PO}_4^{3-}\text{-P}$  levels, while the other two zones were marked with higher sand and nutrients. Shastri stations were marked by high salinity, DO and low levels of nutrients. Salinity and nutrients were the important factors influencing macrobenthic structure at Kundalika and Vashishti (Figs 5a and b).

### **3.6 Degree of similarity between reference conditions**

Fig. 6 represents the percentage similarity in EcoQS obtained based on different RCs in each estuary. Although the RCs were based on different criteria, the degree of similarity in assigning an EcoQS was very high. The Kundalika showed a considerable variation among the RCs, wherein RCI and RCII (default M-AMBI) differed from the RCIII and RCIV (zone-wise M-AMBI) but resembled RCV (virtual M-AMBI). In contrast, the Vashishti and Shastri showed minor variation among the various RCs, signifying uniform EcoQS. The acceptable categories (high and good) were >50% in Kundalika and >80% in the other two estuaries. It was also observed that Vashishti and Shastri did not have any 'poor' and 'bad' categories irrespective of the RC type set.

## 4 Discussion

Tropical estuaries are complex ecosystems whose inherent characteristics undergo major fluctuations due to an assortment of natural and anthropogenic factors. In estuaries, salinity is a key responsible factor for structuring species community and it is difficult to distinguish whether the species are structured due to natural or anthropogenic stressors (estuarine quality paradox) (Dauvin 2007). The salinity zonation criterion gave clarity on the various habitats and has proved to be a promising approach in estuaries (Borja et al. 2012). As the three estuaries differed in the environmental factors and inherent species complexes, the application of different RCs was imperative to gauge which was most effective in EcoQS assignment.

### 4.1 Deriving reference conditions for establishing 'High' status

For the successful application of benthic indices in an estuary, the comparison has to be made with reference conditions (RCs) that denote pristine conditions unaffected by any kind of human pressures. Such immaculate areas do not exist for present day Indian estuaries (Feebarani et al. 2016). Additionally, reliable historical data is also lacking in the Indian milieu. Although for the AMBI calculation the RCs are not required and the index is proven to give efficient results, its application in the inner areas of estuary can give inaccurate results owing to various constraints (Borja and Muxika, 2005). Hence, an improved alternative will be to apply the multi-metric approach i.e. M-AMBI which incorporates species diversity and richness metrics in addition to AMBI values. M-AMBI is estimated based on the ratio of the above parameters between a given site and theoretical RC (Leitner et al. 2021). There are many methods available for derivation of RCs, however, all the methods consist of the same series of steps and differs only in detail (Birk et al. 2013). In this study, we have used five RCs which are based on different criteria used by several authors over the years. When the M-AMBI was developed (Muxika et al. 2007), the RCs were set by taking into account the highest richness and diversity values and low AMBI value which was applied for the whole area. This is the default RC (RCI in Table 1) derived from the software itself. These values of richness and diversity derived from the software were based on the present-day scenario with its incumbent impacts, and thus may not serve as an ideal threshold for the high status. Cai et al. (2014) introduced the concept of increasing the highest richness and diversity values to 15% for the whole area. Hence, we set RCII by increasing the values to 15% for each estuary. However, considering that upper low saline zones have naturally low diversity, using the RCs established for the whole area can result in erroneous classifications (Dias et al. 2018). The transitional waters are highly complex ecosystems and the zonation patterns exist due to the variable salinities which

segregate the macrobenthic assemblages spatially (Chainho et al. 2007). Hence, establishing RCs for each zone became popular (Bald et al. 2005, Borja et al. 2004, Muxika et al. 2007) which is reasonable especially for estuaries that are subjected to high spatial heterogeneity. The RC set for each estuarine zone was referred to as RCIII. Given that all the zones were impacted to some extent, it might not be advisable to use the actual values for setting RCs. Hence, RCIV was computed using the criterion of increasing the highest richness and diversity to 15% for each salinity zone (Mulik et al. 2017). This approach, which has been widely used in various ecosystems, was already applied in the previous study conducted by Dias et al. (2018) in the Kundalika along with the other indices and the EcoQS achieved were relatively satisfactory. However, the same approach might not work in other estuaries, as every estuary is different and unique in its nature. Following Santibañez-Aguascalientes et al. (2020), the virtual RC was derived (RCV) wherein the EGIV and EGV species are removed from the dataset and calculated using the 95<sup>th</sup> percentile for richness and diversity and 5<sup>th</sup> percentile for AMBI. From the various RC derivation methods available, several authors suggested applying RCs that are already derived for other geographical areas (European Commission 2003). However, there is lack of consensus in using these, as RCs vary with each eco-region due to fluctuating natural conditions and intrinsic characteristics within habitats (Hess et al. 2020) and using the same RCs for all the eco-regions seem impractical (Occhipinti-Ambrogi et al. 2009). Hence, based on the merits and de-merits of already available and feasible methods for RCs, five best suitable ones were chosen for this study.

## **4.2 Environmental background**

Perturbations in abiotic characteristics were more evident in the poly-meso-oligohaline zones of Kundalika and Vashishti. The high nutrient concentrations were clearly of anthropogenic origin due to the industrial and domestic effluents being released near these zones (Dias et al., 2018, NIO 2018). The impact of these pollutants was low towards the euhaline zones due to the very good assimilative capacity of Vashishti and excellent flushing nature of Kundalika (NIO 2018; Dias et al., 2018). The Shastri had relatively low concentrations of nutrients and was less impacted by anthropogenic stressors as it had no known point source discharges (NIO 2018). From the comparisons of abiotic variables (Table 2), it can be inferred that the Kundalika was the most impacted, Vashishti was moderately stressed and Shastri was the less affected.

## **4.3 Macrobenthic assemblage structure**

Species diversities were highest in euhaline zones and declined subsequently towards the inner estuary which is typical for tropical estuaries (Mulik et al. 2020a). The univariate measures

provided valuable insights in the species distributional trends corresponding to each estuary. The Kundalika was highly stressed as compared to other two estuaries displaying low diversity in the oligohaline zone. The species varied across the zones of each estuary depending on their sensitivities to the estuarine characteristics and perturbations. The opportunistic EGIV was all pervasive in all the zones of Kundalika, with major dominance in the oligohaline zone due to presence of *Namalycastis indica*. *N. indica* is known to occur in low saline disturbed areas and are highly tolerant to pollutants (Glasby 1999, Mulik et al. 2020b). The other zones of Kundalika were dominated by opportunistic spionid species that are known to proliferate in stressed conditions (Çinar et al. 2012). However, the euhaline zone had other diverse groups belonging to the sensitive species. Amphipods proliferated at the poly-mesohaline (*Corophium volutator*; EGIII) and oligohaline (*Eriopisella sechellensis*; EGI) zones of Vashishti. These species are known to be highly tolerant to pollutants and to occur even in low saline areas (Wongkamhaeng et al. 2009; De-Bastos and Hill, 2016). The overall dominance of amphipods in Vashishti might be due to its functional characteristics of avoiding the polluted sediment and choosing desirable sediment enough for thriving (Okladen et al. 1984). Additionally, the availability of ample food sources might be another reason for the proliferation of the amphipod species, as this estuary had additional inputs from the adjoining tributaries. The euhaline zone of Shastri was dominated by the presence of sensitive EGI species signifying that this zone was conducive for the subsistence of sensitive macrobenthic species as compared to the other estuaries.

#### **4.4 Ecological Quality Status Assessment**

##### **4.4.1 AMBI**

The mean AMBI exhibited a wide variation in EcoQS classes ranging from good to poor in Kundalika, while the other two estuaries were assigned a uniform 'good' EcoQS at all zones. The aggregate 'good' EcoQS of euhaline zones of all estuaries was valid as this zone had lower nutrient concentrations and was less impacted despite the presence of other anthropogenic activities. AMBI erred in considering the poly-mesohaline zone of Kundalika as 'good' even though this zone was impacted by the effluents, as signified by the high levels of nutrients. However, the error could be considered marginal as the EcoQS varied between adjacent categories ('good' vs 'moderate'), probably as the impact was moderate and not severe as compared to the oligohaline zone. The oligohaline zone was rightly assigned 'poor' status as it was more polluted with stressors and had high abundance of opportunist nereids and oligochaetes. AMBI had overestimated the ecological status in this zone of the previous study conducted by Dias et al. (2018), due to the lack of sensitivity in discriminating polluted areas when naturally stressed species occurred in low saline zones (Hutton

et al. 2015). Despite the presence of high nutrients in the oligohaline and poly-mesohaline zones of Vashishti, the EcoQS was indicated as 'good' which might be due to relatively high occurrence of both tolerant and sensitive species compared to opportunistic forms. Reasons for the proliferation of sensitive groups like amphipods (80%) in this area could be attributed to multiplicity of food sources and possible resilience of species to low intensity pollution due to their functional characteristics (Okladen et al. 1984, Dimitriou et al. 2020). In case of Shastri, the aggregate EcoQS was 'good' owing to the presence of sensitive species (*Astarte montagui*) which were higher than the opportunistics. Overall, the discriminating power of AMBI was fair in terms of assigning an EcoQS.

Despite AMBI being suggested as a universal index for assessing EcoQS for NW Indian estuaries by Mulik et al. (2020a), its discrepancies in low saline zones and anthropogenically less impacted estuaries were also highlighted. In this study too, AMBI worked best in highly impacted estuary (Kundalika) compared to the other two low impacted estuaries (Vashishti and Shastri). The index also worked appropriately in the high saline zones and indicated bias in low saline areas which might be due to the changing estuarine scenarios from time to time. Although, AMBI application in the inner areas of estuary can be ineffective due to low species diversity (Afli et al. 2008, Dias et al. 2018), its use in combination with M-AMBI and expert judgment has been recommended by recent studies. The conclusions of this study re-affirm the same.

#### **4.4.2 M-AMBI**

Among the RCs applied for the computation of M-AMBI, RCI, RCII and RCV gave similar results for Kundalika which could describe the EcoQs with greater accuracy for all zones. Thus, the euhaline zone of Kundalika was assigned 'good' status; poly-mesohaline and oligohaline of Kundalika was 'moderate' and 'bad' respectively. These results were largely satisfactory when verified with the abiotic data and expert judgement considering the pressures in these areas. The oligohaline zone of Kundalika was highly stressed due to the high nutrient concentrations and other anthropogenic impacts; hence the 'bad' EcoQS was expected. The oligohaline zone was highly impacted by high nutrients from the discharge point whereas this effect was milder in the poly-mesohaline zone. The occurrence of second-order opportunists as well as sensitive species in the poly-mesohaline indicated that the impact was not severe; hence the 'moderate' EcoQS stands valid. However, RCI, RCII and RCV were severe in assigning the oligohaline zone as 'bad'. The apt EcoQS for this zone based on the presence of second-order opportunistic nereids and oligochaetes would have been 'poor'.

Contrastingly, RCIII and RCIV overestimated the EcoQS in the poly-mesohaline ('high') and oligohaline ('moderate'). For RCIII and RCIV, highest species diversity and richness for each zone are considered. However, as low saline zones with lower diversities will naturally result in lower RCs, using these as a benchmark can result in overestimation (Hu et al. 2019). Thus, zone-wise estimated RCs are bound to result in misclassification as the index takes into account the highest diversity and richness values which are generally interpreted as signs of good EcoQS (Borja et al. 2013). In a previous study conducted in Kundalika by Dias et al. (2018), the zone-wise RC (RCIV in the present study) gave accurate EcoQS. Although, several variations can occur over the span of couple of years, similar cluster of dominant groups (nereids, spionids, nephtyids, oligochaetes) was observed in both studies and hence, similar EcoQS using the RCIV along the zones was expected. However, it overestimated the poly-mesohaline and oligohaline zones in this study thereby contradicting the results of the previous study in Kundalika. Estuaries undergo tremendous changes with varying perturbation levels which are region-specific, time-specific and the same RC may not give similar results. Overall, the current study revealed that Kundalika showed improvement in the euhaline zone due to reduced pollution impact as compared to the previous study, while the other zones maintained similar deteriorated quality due to the sustained anthropic inputs.

The EcoQS achieved by all the applied RCs in the Vashishti was 'good' in the euhaline and poly-mesohaline zones as these areas were characterized by the presence of both sensitive and tolerant taxa in high numbers. In the oligohaline zone, the EcoQS was overestimated as 'high' by the RCIII, RCIV and RCV. This zone was represented by only one station with dominance of EGI species (>80%). EcoQS based on solo station might not be appropriate especially when the zone-wise RCs (RCIII and RCIV) also depict the same richness and diversity values. As RCV considers only the sensitive species (EGI, EGII and EGIII) for setting a RC, the occurrence of high sensitive species (70%) led to overestimation at this zone. Consequently, 'good' was assigned by RCI and RCII (default RCs) since it took into account the species diversity and richness of the whole estuary. The poly-mesohaline and oligohaline zones fell in the 'good' to 'high' EcoQS category as per AMBI and M-AMBI using different RCs. However, these zones, even though impacted by the discharge point, had higher composition of sensitive and tolerant species. It was apparent that the pollution effects were localized station per se (Table A.1) and probably did not affect the entire zone to the extent that the resident macrofauna was affected. Contrasting results such as this have been reported in other studies as well; reasons attributed included difficulty in identifying contamination patterns based on benthic sensitivities in dynamic complex systems and detecting sublethal stress that was not persistent (Salcedo et al. 2017, Santibañez-Aguascalientes et al. 2020). Tett et al. (2013) emphasised

that taxa facing intermediate disturbances can proliferate in areas as the species there are well adapted due to their resilience potential to stressors.

The euhaline zone of Shastri translated into an accurate 'good' EcoQS for the selected RCs (RCI and RCII) and 'high' EcoQS for RCV. The virtual RCs are known to show undisturbed conditions as only the numbers of sensitive species are taken into account. The overestimation by RCV was because it takes into account only sensitive species and the Shastri had high presence of these (>60%) species which apparently makes 'high' EcoQS achievable.

#### **4.5 Link between benthic indices and environmental parameters**

Both AMBI and M-AMBI (based on all RCI, RCII and RCV) indicated a significant relationship with salinity as well as with nutrients in Kundalika. This occurred as high-saline low-nutrient zones (euhaline) were given acceptable status whereas low-saline high-nutrient zones (poly-meso-oligohaline) were given unacceptable status by the indices. However, RCIII and RCIV did not indicate any relevant correlation with abiotic parameters highlighting their limitations in delineating areas of stress. Values based on RCIII and RCIV were not reflective of the nutrient concentrations in the poly-meso-oligohaline (low saline) zones of the estuary. High M-AMBI values were estimated by these two RCs in euhaline (low nutrient) as well the impacted low saline (high nutrient) zones similarly. This happened as the reference values in these cases were based on salinity zones which inflated the EcoQS values disproportionate to the zonal status.

Thus, these indices could be deemed effective with sufficient sensitive discriminatory power in EcoQS assessment in Kundalika. In other estuaries, none of the RCs showed any correlations except for negative correlation of RCII with  $\text{PO}_4^{3-}\text{-P}$  in Vashishti. In Shastri, only  $\text{C}_{\text{org}}$  was positively correlated with AMBI and none with the M-AMBI RCs. From the above observations, it is clear that the discriminatory power of indices was stronger in estuaries with clear gradient of anthropogenic stress. This trend was observed in other previous estuarine studies as well (Mulik et al. 2020a). However, the reliability or discriminatory power cannot be judged based on correlation analyses alone (Fitch and Crowe, 2010; Dias et al. 2018) as each index possesses inbuilt biases.

#### **4.6 Limitations of reference conditions and future considerations**

Different RCs (theoretical threshold of unimpacted RC) were set for M-AMBI as a mandatory step in order to assign a disturbance class (high to bad). The primary differentiating factors for the selected RCs, notwithstanding the various permutations were (a) default input diversity variables versus those with 15% increment (b) consideration of entire estuary versus zones



and (c) inclusion of only sensitive groups. In this study, the application of different RCs did not translate into better index performance due to a host of perceptible reasons. At the outset, the degree of similarity in EcoQS assignation was very high between the RCs with and without the 15% increment, even if the estuary was considered as a whole (RCI vs RCII) or zone-wise (RCIII and RCV). The justification for the suggestion to increase the highest richness and diversity values by 15% (RCIV) as the best scenario was that the default option was deemed invalid in polluted areas/inner areas for which it was suggested (Forchino et al. 2011, Cai et al. 2014). The possible reason for the similar performance of the M-AMBI regardless of the 15% increase in RC was that the mere application of the increment did not result in substantive change in EcoQS as expected.

The euhaline zones were correctly assigned a constant EcoQS in all the estuaries which was in line with the abiotic data and species composition. However, the EcoQS in the poly-mesohaline zones was mostly overestimated depending on the RC and the estuarine zone as the results did not coincide with the expected EcoQS. Among all the applied RCs, the default RCs (RCI and RCII) seemed to suitably assess the EcoQS in most of the zones. Thus, computing RCs uniformly for the whole estuary worked better than those derived for salinity zones. As the low saline areas such as oligohaline and poly-mesohaline are characterised with low species diversity owing to natural factors, it is obvious that the zone-wise RC values will also be low thus reducing the expected standards considerably. Uniform RCs represent more stringent standards for low saline areas thus underestimating them as was demonstrated in the oligohaline zone of Kundalika ('bad' instead of 'poor') of this study. Conversely, zone-wise derived RCs present lenient standards that often overestimate EcoQS as was observed in the poly-mesohaline zones of Kundalika ('high' instead of 'moderate') and oligohaline of Vashishti ('high' instead of 'good'). Thus, ecological status assignation of low saline zones is challenging due to the 'Estuarine Quality Paradox' (Elliot and Quintino, 2007). In this study AMBI correctly identified the oligohaline zones of the estuaries that were investigated. Although several authors do not recommend the default option of M-AMBI software to be used as RC, it has been used widely to derive RCs in transitional waters (Paganelli et al. 2011 and references therein). The RCV based on the removal of EGIV and EGV species (virtual RC) can give biased results, more so in the inner stretches of estuaries. In this study, application of RCV resulted in the overestimation of oligohaline zones of Vashishti and Shastri ('high' instead of 'good'). This happened due to the natural occurrence of dominant opportunistic species and with the removal of these, the EcoQS results in underestimation or overestimation (Hutton et al. 2015, Paganelli et al. 2011).

The exercise of deriving estuarine EcoQS is particularly exacting and in this study, the following factors were observed to have constrained the efficacy.

(i) species composition and their sensitivity: estuarine species that are well adapted to stress show resilience under different environmental settings, can reduce the robustness of the index making the EcoQS assessment difficult; this was evident in the poly-mesohaline zone of Vashishti where sensitive amphipods thrived in the vicinity of an effluent discharge point resulting in 'good' status despite the stressors (high nutrients) present.

(ii) number of stations occurring in each zone: zones with single station might not predict the potential impact as the value obtained for EcoQS is based on one point in an area (ex., the oligohaline zone of Vashishti which was represented by only a single station);

(iii) degree of impact in an area: the index output might not match the expected EcoQS in moderately or less impacted estuaries that hosts both sensitive and tolerant species giving a vague status, unlike the severely impacted areas with more opportunists clearly resulting in the degraded status.

It can be concluded that the use of different RCs did not improve the M-AMBI performance highlighting that the default option actually worked best. Several authors have adopted the default option successfully in various geographical areas without taking into account the estuarine differences or salinity gradient (Borja et al. 2011). The zone-wise RCs were primarily set based on the assumption that none of the stations could suffice as a reference site (Borja et al. 2012). In the previous study of Kundalika, the zone-wise RC (RCIV) seemed efficient in EcoQS evaluation as then the estuary presented more degraded conditions than the present study.

Berthelsen et al. (2018) after a detailed study on the application of biotic indices in New Zealand estuaries opined that defining reference conditions was difficult but important considering the bioregional and inter estuarine variation. It was apparent from this study that the strategy drawn for deducing a successful RC in a particular geographical region may not be applicable in other areas. Species composition and therefore RCs may differ naturally between ecoregions and habitats necessitating establishment of region specific standards for estuarine habitats prior to ecological health assessment (Borja et al. 2008). The use of benthic indices requires validation in new areas or for first time trials in order to ensure that the outcomes are relevant (Moriarty et al. 2018) specifically when there are no historical datasets or baseline data available for the selected region. For instance, the historical data for Vashishti and Shastri was lacking which makes it difficult to validate the assessments based solely on ecological data. Though these areas were characterised by high diversity

and richness signifying ‘good’ EcoQS with less impact, the physico-chemical data showed a substantial impact in the mid-upper stretches of Vashishti. It is important to note that benthic indicators summarise only the species information into unique values to give a disturbance class, which may not be sufficient to correctly assess impact (Dreujou et al. 2021). Thus, before the application of M-AMBI, accurately designed area-specific RCs should be set by considering all the limitations which requires further research with long-term data. Reliance based solely on ecological figures that are data-driven can give a biased or inaccurate view of the estuarine health status which can compromise management strategies for ecological betterment. Therefore, it is recommended that EcoQS assignation for tropical estuaries in particular, be conducted using an approach that combines indicators such as AMBI, M-AMBI with proven RCs and professional judgment.

## **Conclusion**

The present study conducted a detailed EcoQS assessment using AMBI and M-AMBI based on five different RCs for three tropical estuaries. The chosen estuaries had differential perturbations i.e relatively, Kundalika was highly impacted, Vashishti was moderately impacted and Shastri was less impacted. Chemical data indicated that the euhaline zones of all estuaries were relatively less affected by pollutants whereas the signatures of anthropogenic stressors were evident in the poly-meso-oligohaline zones. Both AZTI’s indices and all applied RCs performed appropriately in the euhaline zones but discrepancies in EcoQS assignation were mostly observed in the anthropogenically impacted low saline zones of the estuaries. This study highlighted that setting appropriate RCs is a crucial step in EcoQS assessments as M-AMBI either underestimated or overestimated in many zones. Application of oft-applied RCs did not result in expected improvement of EcoQS; conversely the default RC i.e. lowest AMBI and highest diversity and richness values in the dataset, worked the best among those tested. A major conclusion was that caution requires to be exercised whilst applying the RCs that were deemed successful elsewhere, as the efficacy may not be mirrored between geographical zones and habitat types. Estuaries with sharper pollution trends were easily categorised whereas levels of ambiguity in classification were higher in moderately/low polluted estuaries. Estuarine quality paradox was a major deterrent to effective performance of indices in low saline zones which may be overcome by using the strategy of applying a combination of AZTI’s indices (AMBI and M-AMBI) with appropriate RC and expert judgment for successful evaluation.

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Table 1 Different reference conditions used for M-AMBI calculation in the three estuaries. For RC I, RCII andRCV the same values were used for the whole estuary irrespective of zones; for RCIII and RCIV, values are different for each zone. (For descriptions of RCs refer Fig. 2and section 2.3).

<b>Zone</b>	<b>Metric</b>	<b>RCI</b>	<b>RCII</b>	<b>RCIII</b>	<b>RCIV</b>	<b>RCV</b>
<b>Kundalika</b>						
Euhaline	<i>AMBI</i>	0.5	0.5	0.5	0.5	0.50
	<i>H'</i>	4.07	4.68	4.07	4.68	3.87
	<i>S</i>	33	38	33	38	27
Poly-mesohaline	<i>AMBI</i>			2.58	2.58	
	<i>H'</i>			2.52	2.89	
	<i>S</i>			8	9	
Oligohaline	<i>AMBI</i>			4.5	4.5	
	<i>H'</i>			1.39	1.60	
	<i>S</i>			7	8	
<b>Vashishti</b>						
Euhaline	<i>AMBI</i>	0.29	0.29	0.95	0.95	0.27
	<i>H'</i>	3.79	4.36	3.72	4.28	3.42
	<i>S</i>	30	35	22	25	19
Poly-mesohaline	<i>AMBI</i>			0.29	0.29	
	<i>H'</i>			3.79	4.36	
	<i>S</i>			30	35	
Oligohaline	<i>AMBI</i>			0.99	0.99	
	<i>H'</i>			3.16	3.63	
	<i>S</i>			21	24	
<b>Shastri</b>						
Euhaline	<i>AMBI</i>	0.31	0.31			0.31
	<i>H'</i>	3.99	4.59			3.62
	<i>S</i>	38	44			30

Table 2 Average and standard deviation of physico-chemical variables according to the salinity zones in the three estuaries. Eu=euhaline, Po-Me=poly-mesohaline, Ol=oligohaline

Estuary Zones Stations	Kundalika			Vashishti			Shastri
	Eu 1-6	Po-Me 7-8	Ol 9-10	Eu 1-4	Po-Me 5-9	Ol 10	Eu 1-6
Salinity	35.2±0.9	13.7±7.1	0.7±0.9	35.1±1.0	17.4±8.0	3.5±1.8	35.1±1.2
DO ( $\mu\text{mol l}^{-1}$ )	183.3±9.4	101.6±32.5	141.4±65.0	193.0±13.9	136.6±31.9	159.4±35.4	188.3±12.9
PO <sub>4</sub> <sup>3-</sup> -P ( $\mu\text{mol l}^{-1}$ )	2.9±2.5	5.6±2.3	7.2±4.3	1.3±0.4	1.0±0.6	0.9±0.9	0.6±0.3
NO <sub>2</sub> <sup>-</sup> -N ( $\mu\text{mol l}^{-1}$ )	1.4±0.9	7.8±2.5	4.4±4.6	1.9±1.0	8.7±4.1	5.3±1.3	0.7±0.7
NO <sub>3</sub> <sup>-</sup> -N ( $\mu\text{mol l}^{-1}$ )	13.0±8.5	31.6±9.1	21.8±15.5	6.7±2.3	26.0±7.4	15.0±1.6	3.1±2.3
NH <sub>4</sub> <sup>+</sup> -N ( $\mu\text{mol l}^{-1}$ )	3.8±1.7	24.1±4.7	19.5±6.8	2.1±1.8	9.8±8.9	20.4±5.6	1.6±1.2
SS ( $\text{mg l}^{-1}$ )	156.3±30.3	180.5±96.1	143.8±53.8	95.3±45.0	53.3±61.6	25.0±0.0	19.9±5.9
Sand (%)	29.3±20.7	15.6±5.1	55.0±49.1	37.5±43.7	69.3±24.7	49.6±5.7	35.8±33.0
Silt (%)	52.6±25.3	51.4±17.9	22.8±24.5	55.4±40.9	22.7±20.5	41.1±8.4	50.1±35.1
Clay (%)	17.9±15.9	33.0±18.1	22.2±24.6	7.1±5.3	8.2±5.6	9.3±2.8	14.2±16.7
C <sub>org</sub> (%)	1.9±0.9	2.8±0.8	1.6±1.2	2.4±1.0	2.3±1.1	3.5±0.4	1.8±1.1

Table 3 Average and standard deviation of univariate indices according to the salinity zones in the three estuaries. Eu=euhaline, Po-Me=poly-mesohaline, Ol=oligohaline

Estuary Zones	Kundalika			Vashisti			Shastri
	Eu	Po-Me	Ol	Eu	Po-Me	Ol	Eu
S	18±7.8	7±1.2	3±3.0	15±5.4	16±5.9	20±2.1	27±9.1
N	593±511.7	723±749.7	808±640.1	480±269.4	2345±2274.4	3425±715.9	2743±2050.9
d	2.6±0.9	1.1±0.3	0.2±0.4	2.3±0.8	2.1±0.8	2.3±0.3	3.3±0.9
J'	0.8±0.1	0.7±0.2	0.5	0.8±0.1	0.6±0.2	0.6±0.2	0.7±0.1
ES(100)	14.9±4.8	6.5±1.5	2.2±2.4	13.7±4.6	12.1±3.8	11.1±3.7	16.3±4.2
H'(log2)	3.2±0.5	2.0±0.5	0.3±0.7	3.1±0.8	2.5±0.9	2.5±0.9	3.0±0.7
1-λ'	0.8±0.1	0.6±0.1	0.1±0.2	0.8±0.1	0.7±0.2	0.7±0.2	0.7±0.1
Δ	71.6±9.0	52.8±12.4	10.7±21.4	64.3±8.5	57.9±17.8	60.4±21.4	66.8±11.0
Δ*	84.8±6.1	81.1±9.7	23.5±47.0	79.8±9.0	86.4±10.3	81.7±10.2	88.6±6.8
Δ+	89.3±3.5	78.2±3.1	18.6±37.1	80.2±5.8	82.4±6.3	87.4±4.2	89.1±2.9
Λ+	1583.1±710.8	545.7±77.1	130.0±260.0	1213.3±455.1	1347.8±544.7	1709.0±267.3	2362.4±792.0

Table 4 List of species in the three estuaries 1-20 201-50 501- 800 >800 ind.m<sup>2</sup>

EG	Species	Kundalika			Vashishti			Shastri	EG	Species	Kundalika			Vashishti			Shastri
		Eu	P-M	O	Eu	P-M	OI	Eu			Eu	P-M	OI	Eu	P-M	OI	Eu
I	<i>Palaenotus chrysolepis</i>								III	<i>Notomastus</i> sp.							
I	<i>Aricidea</i> sp.								V	<i>Parheteromastus tenuis</i>							
I	<i>Aricidea longobranchiata</i>								I	Maldanidae							
I	<i>Pherusa</i> sp.								III	<i>Sternaspis scutata</i>							
III	<i>Paraonis gracilis gracilis</i>								II	<i>Perioculodes longimanus</i>							
III	<i>Paraonides lyra capensis</i>								I	Aoridae							
II	<i>Scalisetosus</i> sp.								I	<i>Grandierella</i> sp.							
II	<i>Lepidonotus tenuisetosus</i>								I	<i>Gammaropsis</i> sp.							
II	<i>Lepidonotus carinulatus</i>								I	<i>Gammaropsis nitida</i>							
II	<i>Harmothoe serrata</i>								I	<i>Cheiriphotis</i> sp.							
II	<i>Harmathoe dictyophora</i>								I	<i>Cheiriphotis trifucatus</i>							
I	<i>Diopatra monroi</i>								I	<i>Cheiriphotis megacheles</i>							
II	<i>Diopatra hupferiana monroi</i>								III	<i>Podocerus brassilensis</i>							
I	<i>Diopatra gilchristi</i>								I	<i>Paracaprella</i> sp.							
I	<i>Diopatra cuprea cuprea</i>								II	<i>Caprella</i> sp.							
II	<i>Onuphis holobranchiata</i>								I	<i>Melita</i> sp.							
III	<i>Trochochaeta</i> sp.								I	<i>Ampelisca</i> sp.							
IV	<i>Haploscoloplus kergulensis</i>								I	<i>Ampelisca spinipes</i>							
I	<i>Scolaricia capensis</i>								I	<i>Ampelisca aequicornis</i>							
II	<i>Terebellides stroemii</i>								I	<i>Ampelisca typica</i>							
I	<i>Lanassa capensis</i>								I	<i>Idunella chilensis</i>							
III	<i>Isolda pulchella</i>								I	<i>Idunella picta</i>							
II	<i>Eunice</i> sp.								I	<i>Urothoe pulchella</i>							
II	<i>Phyllodoce capensis</i>								III	<i>Corophium</i> sp.							
IV	<i>Cossura coasta</i>								III	<i>Corophium volutator</i>							
III	<i>Sigambra parva</i>								I	<i>Eriopisella</i> sp.							
IV	<i>Namalycastis senegalensis</i>								I	<i>Eriopisella sechellensis</i>							
IV	<i>Namalycastis indica</i>								I	<i>Erichthonius</i> sp.							
IV	<i>Dendronereis arborifera</i>								I	<i>Cerapus</i> sp.							
I	<i>Neanthes glandicincta</i>								I	<i>Limnodriloides</i> sp.							
II	<i>Ceratonereis mirabilis</i>								V	<i>Limnodrilus hoffmeisteri</i>							
III	<i>Leonnates decipiens</i>								V	Enchytraeidae							
III	<i>Leonnates persicus</i>								V	<i>Tubificoides pseudogaster</i>							
II	<i>Micronereid capensis</i>								V	<i>Pristina</i> sp.							
III	<i>Nereis</i> sp.								I	<i>Sphenia binghami</i>							
II	<i>Nephtys</i> sp.								I	<i>Irus</i> sp.							
I	<i>Nephtys dibranchis</i>								II	<i>Myrtea spinifera</i>							
II	<i>Nephtys paradoxa</i>								I	<i>Venus</i> sp.							
II	<i>Nephtys lyrochaeta</i>								I	<i>Arca nodulosa</i>							
I	<i>Poecilochaetus serpens</i>								III	<i>Glycymeris glycymeris</i>							
II	<i>Euchone</i> sp.								I	<i>Astarte montagui</i>							
II	<i>Chone filicaudata</i>								I	<i>Gari</i> sp.							
II	<i>Glycera</i> sp.								I	<i>Gari depressa</i>							
II	<i>Glycinde capensis</i>								I	<i>Fabulina fabula</i>							
II	<i>Glycinde kameruniana</i>								III	<i>Cardiolucina quadrata</i>							
II	<i>Glycera longipinnis</i>								I	<i>Lucinoma borealis</i>							
II	<i>Glycera natalensis</i>								III	<i>Abra</i> sp.							
II	<i>Goniada maculata</i>								III	<i>Abra nitida</i>							
II	<i>Goniada emerita</i>								I	<i>Abra prismatica</i>							
II	<i>Lumbrineris meteorana</i>								I	<i>Acanthocardia</i> sp.							
II	<i>Lumbrineris heteropoda</i>								NA	Potamididae							
II	<i>Ninoe</i> sp.								NA	Nassariidae							

IV	<i>Prionospio</i> sp.				NA	Architectonicidae			
IV	<i>Prionospio sexoculata</i>				I	<i>Neripteron violaceum</i>			
IV	<i>Prionospio cirrifer</i>				II	Naticidae			
IV	<i>Prionospio cirrobranchiata</i>				II	<i>Clithon</i> sp.			
IV	<i>Paraprionospio</i> sp.				III	Amphiporidae			
IV	<i>Paraprionospio patiens</i>				I	Aspidosiphonidae			
III	<i>Aonides</i> sp.				I	Golfingiidae			
IV	<i>Polydora</i> sp.				I	Sipunculidae			
I	<i>Magelona cincta</i>				I	<i>Phascolion</i> sp.			
I	<i>Magelona crenulifrons</i>				II	<i>Campylaspis</i> sp.			
IV	<i>Tharyx</i> sp.				I	<i>Iphinoe</i> sp.			
IV	<i>Tharyx filibranchia</i>				I	<i>Amphipholis</i> sp.			
IV	<i>Cirratulus</i> sp.				I	<i>Amphiodia</i> sp.			
III	<i>Aphelochaeta filiformis</i>				II	<i>Amphiura</i> sp.			
IV	<i>Caulleriella acicula</i>				III	<i>Viscosia</i> sp.			
I	<i>Pectinaria</i> sp.				NA	<i>Baharilana</i> sp.			
I	<i>Pectinaria papillosa</i>				II	<i>Phoronis</i> sp.			
V	<i>Capitella capitata</i>				III	<i>Apeudes</i> sp.			
IV	<i>Heteromastus filiformis</i>				II	Anthozoa			
III	<i>Mediomastus</i> sp.				II	<i>Echiurus echiurus</i>			
IV	<i>Mediomastus capensis</i>								

Table 5: Spearman correlation coefficients ( $p < 0.05$ ) between indices and environmental variables in the three estuaries. RC=reference condition.

	Salinity	DO	PO <sub>4</sub> <sup>3-</sup> -P	NO <sub>2</sub> <sup>-</sup> -N	NO <sub>3</sub> <sup>-</sup> -N	NH <sub>4</sub> <sup>+</sup> -N	SS	Sand	Silt	Clay	C <sub>org</sub>
<b>Kundalika</b>											
AMBI	-0.8					0.58					
RC I	0.83		-0.49	-0.52		-0.74					
RC II	0.83		-0.49	-0.52		-0.74					
RC IV		-0.46									
RC V	0.83		-0.49	-0.52		-0.74					
S	0.6		-0.58			-0.58					
d	0.69		-0.58	-0.46		-0.63					
J'	0.62			-0.58		-0.59				-0.68	
H'	0.75		-0.55	-0.49	-0.45	-0.74					
1-λ'	0.75		-0.5	-0.48		-0.77		0.51			
ES (100)	0.64		-0.58			-0.62					
Δ	0.76		-0.54	-0.49		-0.8		0.45			
Δ*	0.85		-0.61	-0.53	-0.58	-0.76					
Δ+	0.6		-0.59		-0.48	-0.58					
<b>Vashishti</b>											
AMBI	0.47										
RC II			-0.45								
S								0.45	-0.46		
N	-0.67	-0.58		0.72	0.54	0.48	-0.56				
d								0.46	-0.46		
J'	0.53	0.54		-0.58							
Δ*		-0.53									
Δ+			-0.55								
<b>Shastri</b>											
AMBI											0.63

## Figure captions

Fig 1 Study area map showing the station locations in the three estuaries.

Fig. 2 Schematic representation of the steps involved in the assessment of EcoQS.

Fig 3 Composition of ecological groups occurring at each zone in the three estuaries.

Eu=euhaline, Po-Me=poly-mesohaline, Ol=oligohaline.

Fig 4 Benthic indices (AMBI and M-AMBI) using five reference conditions in the estuaries.

Fig 5 Canonical correspondence analyses triplots of macrobenthic species and abiotic variables at the different stations of the three estuaries: Kundalika (a); Vashishti (b) and Shastri (c).

→: environmental variables: Salinity, DO,  $\text{PO}_4^{3-}\text{-P}$ ,  $\text{NO}_2^{-}\text{-N}$ ,  $\text{NO}_3^{-}\text{-N}$ ,  $\text{NH}_4^{+}\text{-N}$ , SS, Sand, Silt, Clay and  $\text{C}_{\text{org}}$ ; Blue colour indicates macrobenthic species: *Nam oua*: *Namalycastis ouanaryensis*, *Nam ind*: *Namalycastis indica*, *Nea gla*: *Neanthes glandicineta*, *Nep par*: *Nephtys paradoxa*, *Pri sp.*: *Prionospio sp.*, *Pri cir*: *Prionospio cirrifera*, *Par sp.*: *Paraprionospio sp.*, *Par pat*: *Paraprionospio patiens*, *Med sp.*: *Mediomastus sp.*, *Par ten*: *Parheteromastus tenuis*, *Amp aeq*: *Ampelisca aequicornis*, *Tub pse*: *Tubificoides pseudogaster*, *Aps sp.*: *Apseudes sp.*, *Gly nat*: *Glycera natalensis*, *Pec pap*: *Pectinaria papillosa*, *Med cap*: *Mediomastus capensis*, *Mal*: Maldanidae (gen. sp.), *Per lon*: *Perioculodes longimanus*, *Gam sp.*: *Gammaropsis sp.*, *Gam nit*: *Gammaropsis nitida*, *Cor vol*: *Corophium volutator*, *Eri sec*: *Eriopisella sechellensis*, *Iru sp.*: *Irus sp.*, *Abr sp.*: *Abra sp.*, *Cos coa*: *Cossura coasta*, *Tha fil*: *Tharyx filibranchia*, *Cap cap*: *Capitella capitata*, *Amp spi*: *Ampelisca spinipes*, *Che meg*: *Cheiriphotis megacheles*, *Ast mon*: *Astarte montagni*, *Gol*: Golfingiidae (gen. sp.).

Fig 6 Similarity percentage of EcoQS obtained between the reference conditions in estuaries.



Title: Ecological quality status assessment of tropical estuaries with benthic indices using differently derived reference conditions

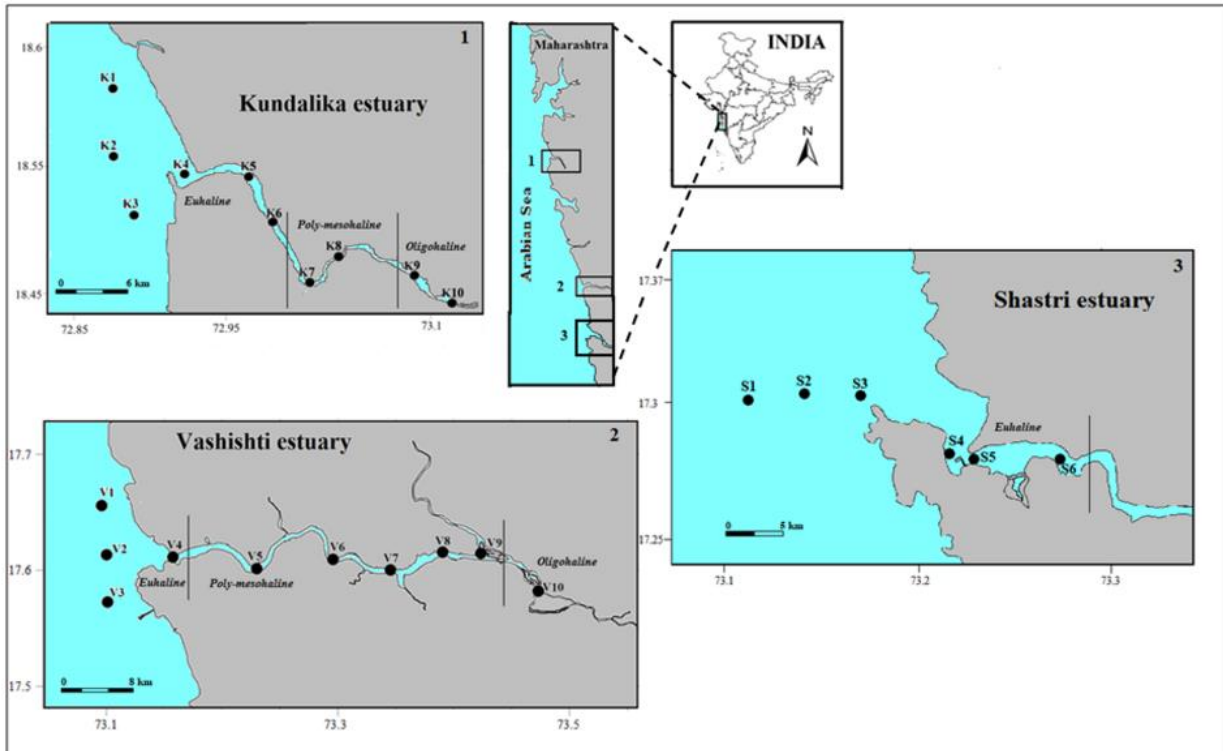


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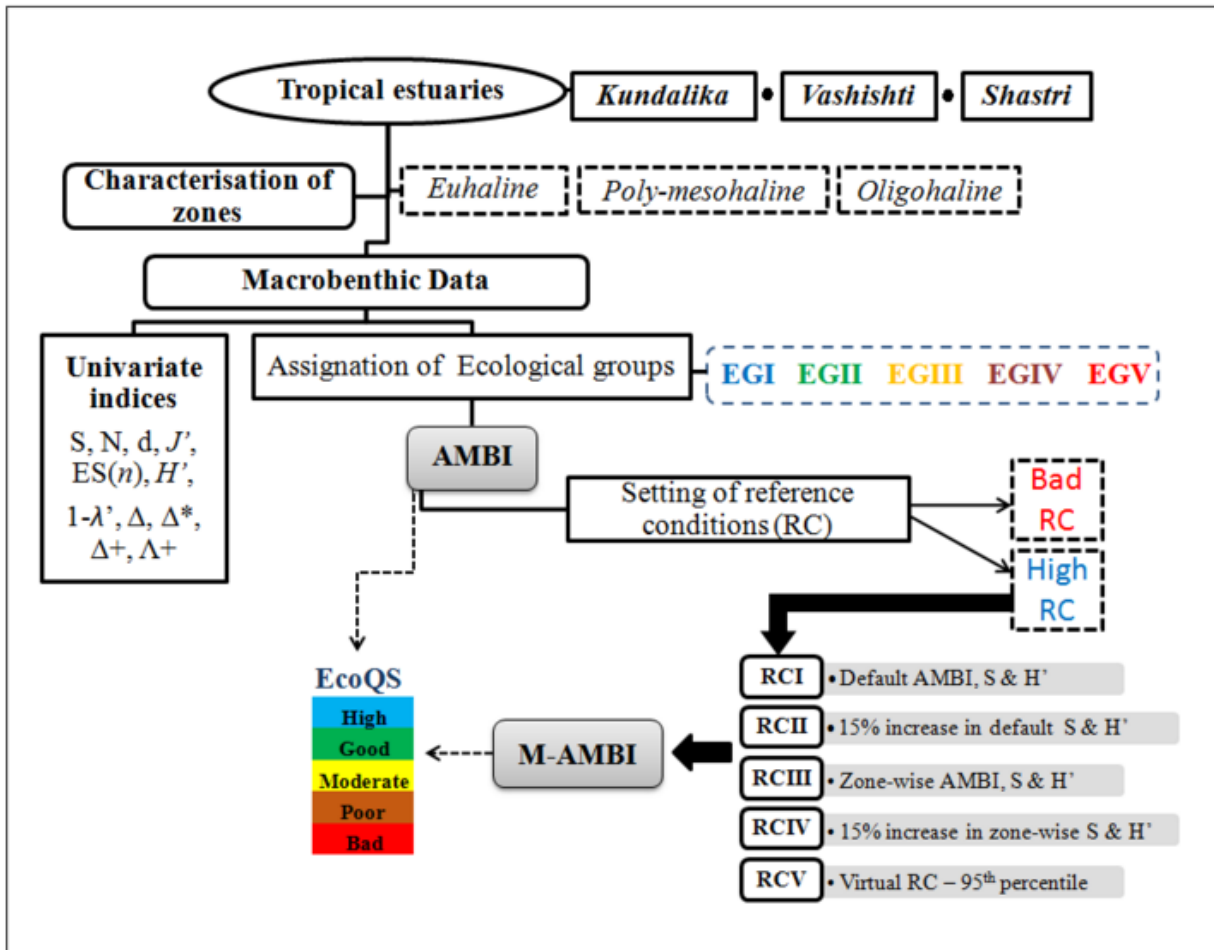


Fig. 2 Schematic representation of the steps involved in the assessment of EcoQS.

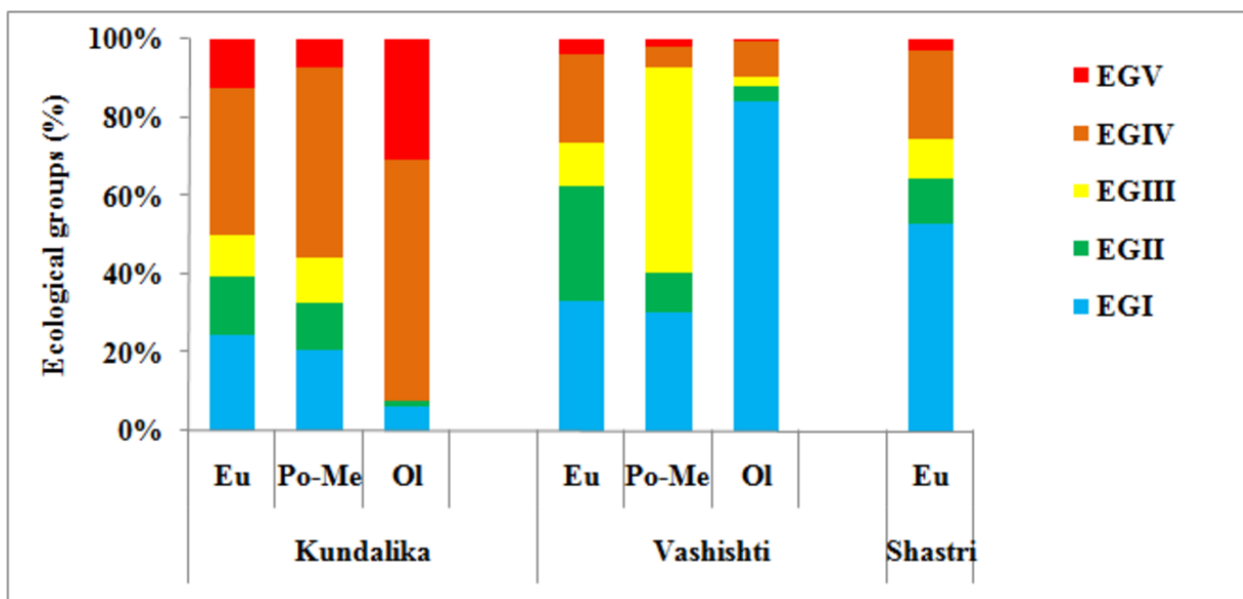


Fig. 3 Composition of ecological groups occurring at each zone in the three estuaries. Eu=euhaline, Po-Me=poly-mesohaline, Ol=oligohaline.

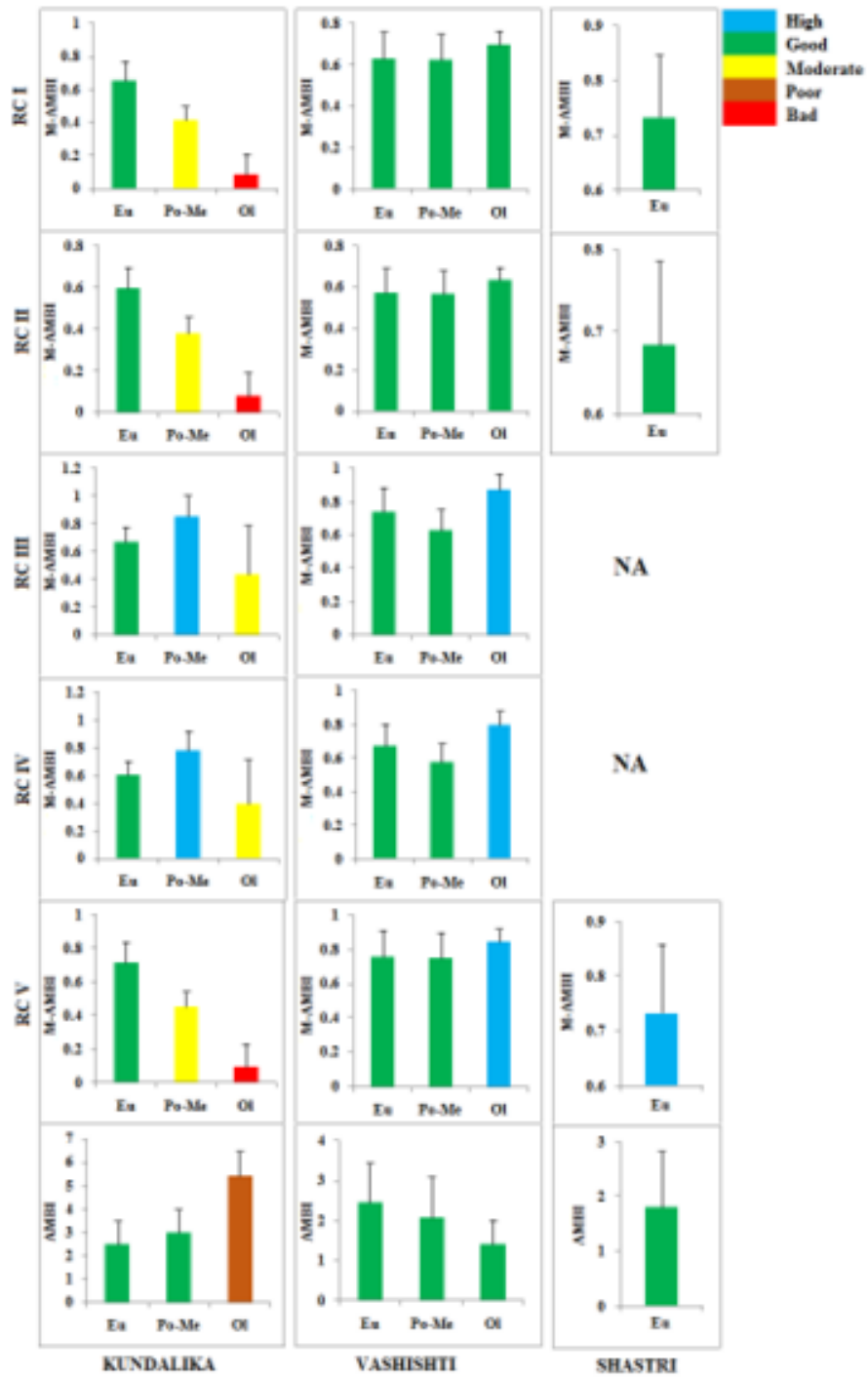


Fig. 4 Benthic indices (AMBI and M-AMBI) using five reference conditions in the estuaries.

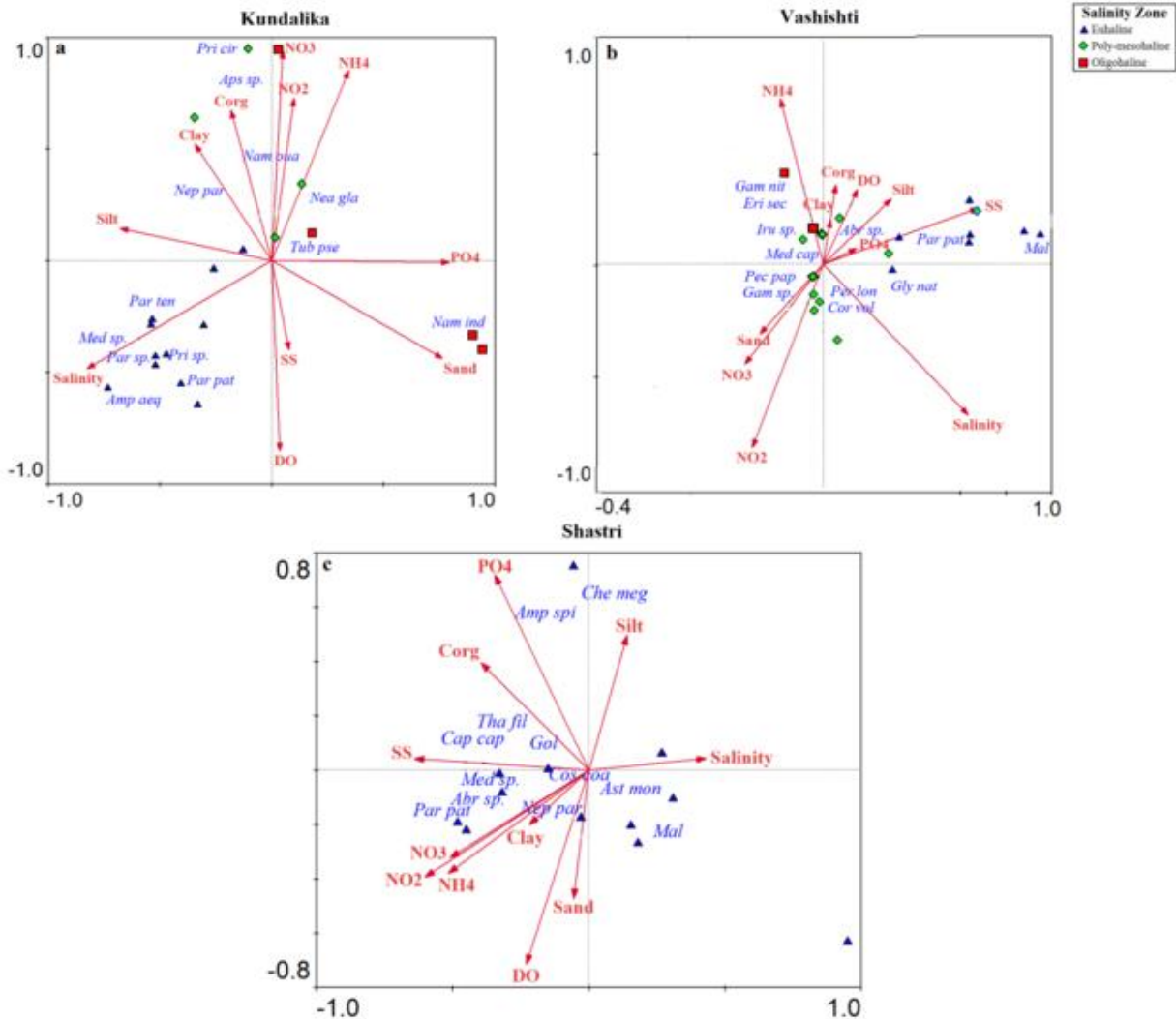


Fig. 5 Canonical correspondence analyses triplots of macrobenthic species and abiotic variables at the different stations of the three estuaries: Kundalika (a); Vashishti (b) and Shastri (c).

→: environmental variables: Salinity, DO, PO<sub>4</sub><sup>3-</sup>-P, NO<sub>2</sub><sup>-</sup>-N, NO<sub>3</sub><sup>-</sup>-N, NH<sub>4</sub><sup>+</sup>-N, SS, Sand, Silt, Clay and C<sub>org</sub>; Blue colour indicates macrobenthic species: *Nam oua*: *Namalycastis ouanaryensis*, *Nam ind*: *Namalycastis indica*, *Nea gla*: *Neanthes glandicineta*, *Nep par*: *Nephtys paradoxa*, *Pri sp.*: *Prionospio sp.*, *Pri cir*: *Prionospio cirrifera*, *Par sp.*: *Paraprionospio sp.*, *Par pat*: *Paraprionospio patiens*, *Med sp.*: *Mediomastus sp.*, *Par ten*: *Parheteromastus tenuis*, *Amp aeq*: *Ampelisca aequicornis*, *Tub pse*: *Tubificoides pseudogaster*, *Aps sp.*: *Apseudes sp.*, *Gly nat*: *Glycera natalensis*, *Pec pap*: *Pectinaria papillosa*, *Med cap*: *Mediomastus capensis*, *Mal*: Maldanidae (gen. sp.), *Per lon*: *Perioculodes longimanus*, *Gam sp.*: *Gammaropsis sp.*, *Gam nit*: *Gammaropsis nitida*, *Cor vol*: *Corophium volutator*, *Eri sec*: *Eriopisella sechellensis*, *Iru sp.*: *Irus sp.*, *Abr sp.*: *Abra sp.*, *Cos coa*: *Cossura coasta*, *Tha fil*: *Tharyx filibranchia*, *Cap cap*: *Capitella capitata*, *Amp spi*: *Ampelisca spinipes*, *Che meg*: *Cheiriphotis megacheles*, *Ast mon*: *Astarte montagni*, *Gol*: Golfingiidae (gen. sp.).

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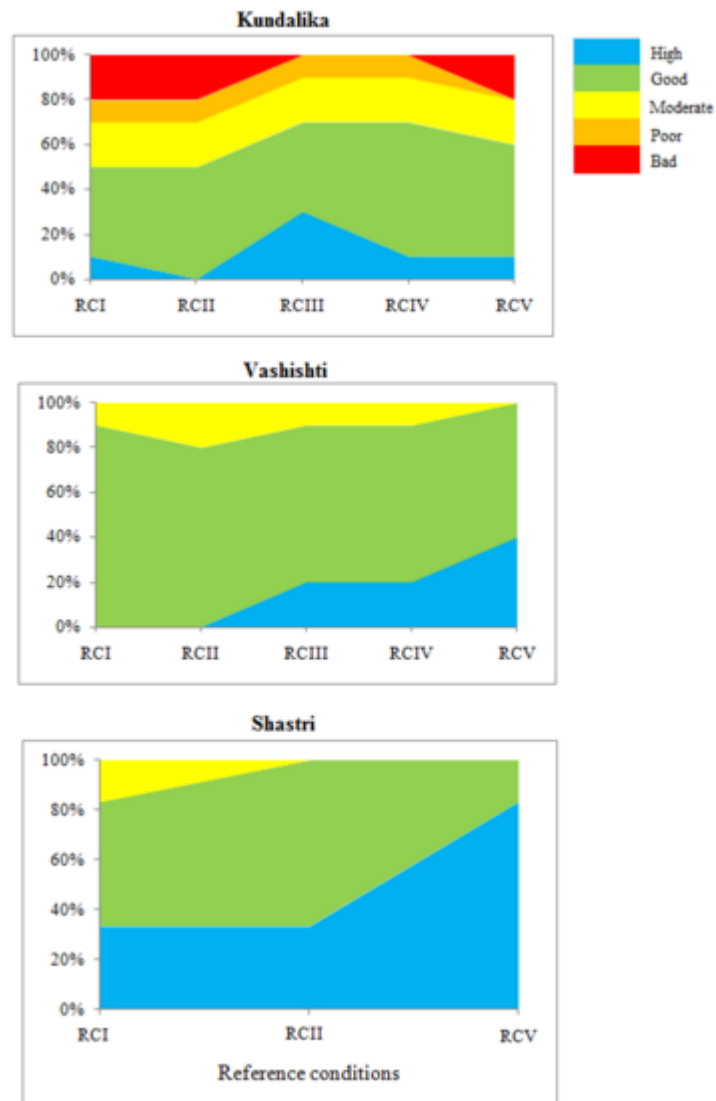


Table A1: Mean values of AMBI and M-AMBI at different stations in the three estuaries. Colors indicate the EcoQS (Blue=High, Green=Good, Yellow=Moderate, Orange=Poor, Red=Bad).

Stations	Kundalika						Vashishti						Shastri			
	AMBI	M-AMBI					AMBI	M-AMBI					AMBI	M-AMBI		
		RCI	RCII	RCIII	RCIV	RCV		RCI	RCII	RCIII	RCIV	RCV		RCIII	RCIV	RCV
1	2.60	0.62	0.57	0.64	0.58	0.68	2.08	0.60	0.55	0.70	0.64	0.72	1.08	0.77	0.70	0.86
2	2.12	0.68	0.62	0.69	0.63	0.74	2.56	0.56	0.51	0.66	0.60	0.67	1.26	0.79	0.72	0.89
3	1.61	0.69	0.63	0.69	0.63	0.75	2.85	0.63	0.58	0.74	0.67	0.76	1.69	0.81	0.73	0.91
4	1.63	0.81	0.74	0.83	0.75	0.88	2.35	0.74	0.67	0.87	0.79	0.88	2.52	0.74	0.67	0.83
5	3.36	0.62	0.56	0.64	0.58	0.67	2.92	0.64	0.58	0.65	0.59	0.77	3.31	0.71	0.64	0.79
6	3.70	0.51	0.46	0.53	0.48	0.55	2.46	0.47	0.43	0.47	0.43	0.57	1.04	0.58	0.53	0.65
7	2.63	0.46	0.42	0.93	0.85	0.50	2.41	0.74	0.67	0.74	0.67	0.89				
8	3.30	0.37	0.34	0.78	0.71	0.40	1.60	0.63	0.57	0.64	0.58	0.76				
9	5.28	0.14	0.13	0.52	0.47	0.15	1.01	0.65	0.59	0.65	0.60	0.78				
10	5.61	0.03	0.03	0.35	0.33	0.04	1.41	0.70	0.64	0.88	0.80	0.84				

Fig A1: Comparison of mean M-AMBI values along with standard deviation between the years 2014 and 2015-16 at each station of Kundalika estuary.

