Addressing a gap in the Water Framework Directive implementation: Rocky shores assessment based on benthic macroinvertebrates

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Highlights

Rocky shore macroinvertebrate communities were assessed along disturbance gradients.
Indices and models were tested along gradients, and compared to MarMAT assessment.
The most efficient indices were combined into a WFD compliant multimetric index.
The multimetric index was able to capture differences across the gradients.
Results were validated using independent data.

Abstract

A gap in the European Water Framework Directive (WFD) is addressed, aiming for the development of an ecological quality status assessment tool based solely on the Biological Quality Element benthic macroinvertebrates from intertidal rocky shores. The proposed Rocky shore Macroinvertebrates Assessment Tool (RMAT) was tested and validated along disturbance gradients (organic enrichment). During the whole process, the response of widely used metrics (e.g. Hurlbert index, Shannon-Wiener index, AZTI's Marine Biotic Index; Bentix biotic index) and models (i.e., metrics combined) was compared to results provided by the Marine Macroalgae Assessment Tool to the same sampling sites.

The RMAT is a multimetric index compliant with the WFD based on the benthic macroinvertebrates community, combining 'abundance' (Hurlbert index) and 'taxonomic composition' (Bentix index using density and biomass data) metrics. It performed well along anthropogenic disturbance gradients, showing ecological quality increasing from close to far away from the disturbance.

The RMAT is a promising tool for rocky shore ecological assessment in the scope of the WFD or other monitoring activities worldwide.

Keywords

Benthic communities; Hard bottom; Organic enrichment; Ecological quality assessment; Water Framework Directive

1. Introduction

The European Water Framework Directive (WFD, 2000) was implemented to 'establish a framework for the protection of inland surface waters, transitional waters, coastal waters and ground waters'. The WFD requires Member States to assess the ecological quality status (EQS) of all water bodies, based on the status of the biological quality elements (BQE) as well as hydromorphological and physical-chemical quality elements. The EQS is determined by the deviation (ecological quality ratio, EQR) that the biological elements exhibit from the expected at undisturbed or nearly undisturbed situations (reference conditions) (WFD, 2000). The WFD specified a five-point scale for water quality, ‘Bad’, ‘Poor’, ‘Moderate’, ‘Good’ and ‘High’; the ‘High status’ is represented by EQR values close to 1, whilst the ‘Bad status’ is expressed by values close to 0.

A major issue in the implementation of the WFD is defining reference conditions. This should be done using historical and monitoring data, modelling or, ultimately, resorting to expert judgement (WFD, 2000). This is largely because historical data is scarce on the pressures impacting ecosystems and the consequent long-term changes (Borja et al., 2012). Also, recent monitoring data may not be comparable due to different methodologies (e.g., sampling and processing) and lack of intercalibration among Member States, further slowing the implementation of the WFD (Poikane et al., 2014). In brief, Member States should reach an agreement on quality standards (e.g., set reference conditions and establish boundaries between EQS classes) so that the different methods produce comparable classifications for each BQE (Birk et al., 2013).

Coastal rocky shores extend to over 80% of the coastline worldwide (Emery and Kuhn, 1982; Granja, 2004). They are important marine habitats with great biodiversity, providing valuable ecosystem services, namely provisioning, regulating and cultural services (e.g., Liquete et al., 2013; Galparsoro et al., 2014). The particular environmental conditions (e.g., wave exposure, tidal regime) of rocky shores add challenges to the ecological status assessment. The intertidal rocky shore is a very harsh environment and biotic communities there are naturally highly variable (Thompson et al., 2002). Difficulties in distinguishing natural from anthropogenic disturbance (e.g., organic enrichment) have often been highlighted (e.g., Crowe et al., 2000; Thompson et al., 2002; Elliott and Quintino, 2007). This hampers the WFD implementation with regard to rocky shores, namely in the development of an ecological assessment tool (e.g., defining reference conditions, setting boundaries between EQS classes). Despite that, rocky shore communities have also often shown to respond to different levels of disturbance (e.g., Bishop et al., 2002; Kraufvelin, 2007; O'Connor, 2013; Cabral-Oliveira et al, 2014; Vinagre et al., 2016a).

For assessment of coastal and transitional waters, several multimetric ecological tools have been developed based on the different BQEs (Birk et al., 2012), combining complementary, metrics to summarize the ecosystem health into a single, and comprehensible value. Also, several biological elements (e.g., macroalgae, phytoplankton) have been intercalibrated among Member States (Poikane et al., 2014). For benthic macroinvertebrates, however, the intercalibration exercise has been undertaken only for the soft sediment habitat, while for hard substratum (i.e., rocky shores) that has not been the case (Borja et al., 2009a). This is because, despite macroalgae and benthic macroinvertebrates being the most suitable BQEs for rocky shore assessment, the tools available are exclusively (Ballesteros et al., 2007; Juanes et al., 2008; Neto et al., 2012; Ar Gall and Le Duff, 2014), or in part (Hiscock et al., 2005; Díez et al., 2012; O'Connor, 2013) based on the macroalgae. Although macroinvertebrates are widely recognized as good indicators of water quality and pollution, to date, attempts to develop an index based exclusively on this BQE (Hiscock et al., 2005; Díez et al., 2012; Orlando-Bonaca et al., 2012) were not totally successful. This was possibly because of the approaches widely used by rocky shore ecologists (e.g., using non-destructive percentage cover instead of destructive samples of density or biomass, or using a low taxonomic resolution). Therefore, a method based specifically on the benthic macroinvertebrates from hard substratum constitutes a gap in the WFD implementation (Birk et al., 2012).

The overall aim of this work was to address that gap in the WFD implementation, and to propose a multimetric index based exclusively on rocky shore macroinvertebrates, the *Rocky shore Macroinvertebrates Assessment Tool* (*RMAT*). The RMAT seems promising for WFD rocky shore quality assessments, and may be a valuable indicator in the scope of other European Directives (e.g., Marine Strategy Framework Directive).

In parallel to the RMAT, an alternative index (alt-RMAT) is presented; this is not as accurate as the former but is quicker and less expensive to apply when time or resources are limited.

2. Methods

2.1. Study sites

The Buarcos (40°10'14.2"N, 8°53'26.7"W) and Matadouro (38°58'31.5"N, 9°25'14.4"W) rocky shores are located in the western Portuguese coast (Fig. 1A) and classified as Exposed and Moderately Exposed Atlantic Coast typologies (TICOR project, Bettencourt et al., 2004; available at http://www.ecowin.org/ticor), respectively.

Along this coast the prevailing current direction is from West-Northwest, and the most frequent wave period and wave height are in the range of 8-12 s and of 1-3 m, respectively. Tide is semidiurnal and the extreme spring tide ranges from 3.5-4 m (Boaventura et al., 2002, Bettencourt et al., 2004).

Both shores are subject to moderate impact from continuous throughout the year runoff of waters crossing urban centres and agricultural land before reaching the shore (Vinagre et al., 2016a, b, 2017).

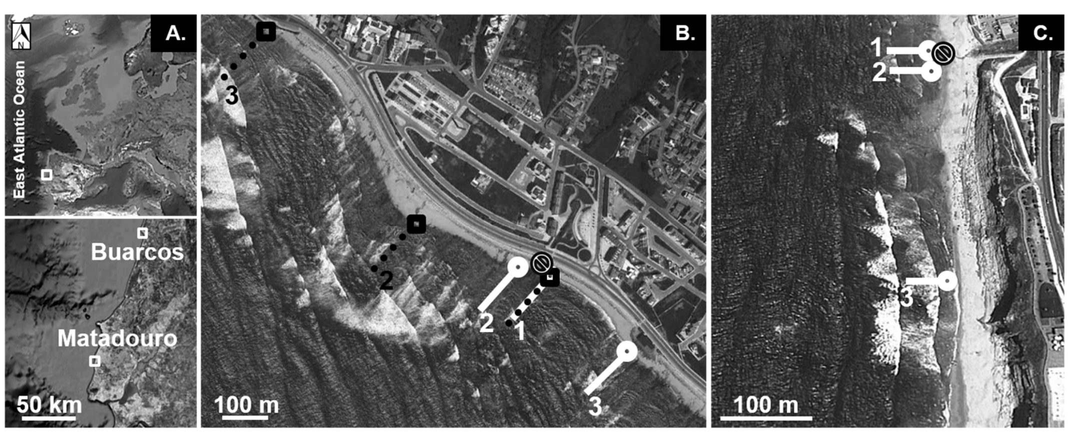

Figure 1. Study sites location: A. Europe and Portugal. B. Buarcos (40°10'14.2"N, 8°53'26.7"W). C. Matadouro (38°58'31.5"N, 9°25'14.4"W). Sampling sites = white circles full line; Validation sites = black squares dotted line; Source of disturbance = ⊘ sign (adapted from Vinagre et al., 2016b).

2.2. Data collection

Eleven ecological indices based on macroinvertebrates were selected from Vinagre et al. (2016b). These were those that performed best along the disturbance gradients at both shores, especially during summer. Summer data (collected during August and September 2011) was used as it was previously found as the better season (comparing to winter) for monitoring activities on rocky shores (Vinagre et al., 2016b, 2017). The indices were calculated using macroinvertebrates' density (ind m^{-2}) and biomass (g AFDW m^{-2}) data, estimated from samples collected at three sites distancing gradually along the disturbance gradient (site 1 closest to the disturbance, site 3 farthest from the disturbance) (Figs. 1B, 1C). Each site was divided into three intertidal zones: upper intertidal (submersed for ~25% of the tide period, ~6h/day); mid intertidal (submersed for ~50% of the tide period, ~12h/day); and lower intertidal (submersed for ~75% of the tide period, ~18h/day). Four random 12 x 12 cm samples were collected at each intertidal zone. This size has been used with success to study anthropogenic disturbance scenarios impacting rocky shore macroinvertebrate communities (e.g., Pais-Costa, 2011; Cabral-Oliveira et al., 2014). Previous to the current work, the authors used Pais-Costa (2011) data to assess the number of replicate samples necessary for stabilization of variability of community parameters (density), and from the six replicates used by Pais-Costa (2011), four were deemed sufficient. Hence, a total of 144 samples (2 sampling events during summer * 2 shores * 3 sites * 3 zones per site * 4 replicates per zone) were used for each index.

The data set included abundance/diversity metrics and indices based on taxonomic composition. In the former group were listed the total biomass of opportunistic taxa [ecological groups (EG) III-V from AZTI's Marine Biotic Index (AMBI, Borja et al., 2000), after update of missing EG (Vinagre et al., 2016a, b)], Margalef index (Margalef, 1968), Hurlbert index (Hurlbert, 1971), Shannon-Wiener index (log_2) (Shannon and Weaver, 1963) and (complement of) Simpson index (Simpson, 1949) (all calculated using biomass data, B_{opp}, d_B, ES_{10}, H'_B and 1-λ'_B, respectively). The last group was composed of the AMBI, MEDiterranean OCCidental index (MEDOCC, Pinedo and Jordana, 2008) and Bentix biotic index (BENTIX, Simboura and Zenetos, 2002), calculated using density: AMBI, MEDOCC and BENTIX, and using biomass: AMBI_B, MEDOCC_B and BENTIX_B, respectively. All indices were calculated per replicate.

2.3. Data analysis

The construction of the proposed multimetric index RMAT was based on two different methods. Method 1 was essential to find the most suitable metrics (i.e., correlating stronger with the disturbance gradient) to integrate the multimetric index (e.g. using multivariate analysis of data). Method 2 was followed in parallel to reinforce the results of Method 1 (e.g., using multiple linear regressions to aid in the selection of the metrics more correlated to the disturbance).

Some analyses may seem redundant (e.g., Method 1 step B. vs step C., Method 1 step G. versus Method 2). However, owing to the novelty of the work, it was necessary to reduce any uncertainty from the results obtained, to assure the indices selected were the most appropriate.

In both methods, the relationship between macroinvertebrate indices and pressure is indirect being tested against the *MarMAT* (*Marine Macroalgae Assessment Tool*) assessment, a WFD compliant tool based on rocky shore macroalgae that has been shown to respond to pressure (Neto et al., 2012).

2.3.1. Developing method 1

All analyses (except boxplots) were performed with PRIMER 6 + PERMANOVA© software (Clarke and Gorley, 2006; Anderson et al., 2008). Boxplots were drawn using Minitab® V.17 (Minitab Inc.) statistical software.

2.3.1.1. Preliminary data set

The data set including the 11 ecological indices was used to visualize the distribution of sampling sites along disturbance gradients, by performing Principal coordinate (PCO) analyses. The Euclidean similarity measure was used in the calculation of similarity matrices, after normalisation of data. The main indices related to that distribution were found looking at principal component analysis (PCA) eigenvalues for the first two axes.

Subsequently, data from each index were analysed separately to assess which were the best candidates to be included in the multimetric index:

A. Significant differences were investigated using permutational multivariate analysis of variance (PERMANOVA; Anderson, 2001), including one fixed factor, ‘Site’ (three levels: sites 1-3). Similarity matrices were calculated as for the PCO analysis. The statistical significance of variance components was tested using 9999 unrestricted permutations of raw data, with a significance level of $\alpha = 0.05$. This was done to verify the indices that could better distinguish among sites along disturbance gradients (together with PCO and PCA analyses);

B. The resemblance in data structure between each macroinvertebrate index and MarMAT was analysed using the RELATE routine (comparative Mantel-type tests on similarity matrices) on the basis of the Euclidean similarity measure (Spearman correlation and 9999 permutations were used). For the invertebrate indices were used values per replicate in the calculation of similarity matrices. For the MarMAT were used values calculated at site level for each shore (for methodological reasons; see Gaspar et al., 2012 and Neto et al., 2012);

C. Relations among macroinvertebrate indices were examined using the Draftsman plot routine with calculation of Pearson correlations). Highly correlated (>0.9) indices were removed from the dataset. In this step, correlations were also calculated between macroinvertebrate indices and the MarMAT, to reinforce the results from RELATE (step B. in this section) (using raw data instead of similarity matrices). Hence, to make the correspondence between the invertebrate indices and MarMAT possible, the same MarMAT value from a site was compared to all samples from that site for each invertebrate index (e.g., MarMAT value of Buarcos site 1 was given to 24 samples of Buarcos site 1 from each invertebrate index; 24 samples from site 1 = 4 replicates * 3 intertidal zones * 2 sampling events during summer).

This was the final data set used in further analysis of Method 1. All variables in this data set were used in the remaining analyses of Method 1, to aid the comparison with results from Method 2.

2.3.1.2. Final data set

After correlated (>0.9) variables been removed, the final data set was used to:

D. Re-analyse the sampling sites' distribution and the indices responsible for it, by performing a second PCO and PCA;

E. Show the indices trends across sites along the gradients, by drawing box-and-whiskers plots (with median values) and mean values (with respective standard deviation) and comparing them with MarMAT;

F. Analyse indices' contribution to differences within and among sites, by performing (i) multivariate Similarity Percentage Analysis (SIMPER) and (ii) univariate SIMPER. Average square distances (ASD) were assessed looking for the indices which showed lower distance within, and higher distance among, sampling sites;

G. Assess the 'best' indices to integrate RMAT, using Distance-based Linear Modelling (DistLM; Anderson, 2004): i) entering all variables, and using ii) backward selection, iii) forward selection, iv) step-wise selection and v) BEST selection – best variable, best two variables and best three variables. The BIC (Bayesian Information Criterion) selection criterion was selected, and 9999 permutations were used;

H. Select the best indices to be included in the multimetric index. The index which performed worst at each analysis (steps A.-C. in section 2.3.1.1, and steps D. and F. in this section), showing poorer relation with the disturbance gradients, received lowest score (of 1), the second worse index got the second lowest score (of 2), and so forth.

From the PCA (step D. in this section), the scores given were calculated as [((2 * PCA1) + PCA2) / 3], to give additional weighing to PCA1. From the multivariate SIMPER (step F.(i) in this section) were used the mean value of all sites scores, and the score from the comparison between sites 1 and 3. From the univariate SIMPER (step F.(ii) in this section) was used the mean value [((2*Between sites 1,3) + Within sites 1,2,3) / 3].

The indices with highest final scores (sum of all analyses scores), reflecting best overall performance, were selected to integrate the RMAT (considering also the indices selected in step G. in this section).

2.3.2. Developing Method 2

In parallel to Method 1, the data set with 11 indices was used together with MarMAT in multiple linear regressions (MLR), using the Brodgar© V2.7.1 (Highland Statistics Ltd.) software, linked to the R-2.9.1 (R Development Core Team) open-source software. This was done to determine which indices (explanatory variables, EVs) were best related to MarMAT (response variable, RV), to complement Method 1 in the selection of 'best' indices to integrate the proposed RMAT. Similarly to what was done in Method 1 (step C. in section 2.3.1.1), to make the correspondence between RMAT and MarMAT possible, all RMAT Buarcos site 1 samples were given the MarMAT value for Buarcos site 1, all RMAT Matadouro site 1 samples were given the MarMAT value for Matadouro site 1, and so forth for the remaining sites at both shores.

To conduct the MLRs, collinearity between EVs was first analysed, looking at variance inflation factors (VIFs). After each test, the metric showing highest VIF was removed from the data set before the next test, until all EV in the data set presented acceptable VIFs (≤5). Simultaneously, interactions between EVs were inferred using conditional scatterplots (Coplots) for the RV against one EV conditioned on another EV. This checked for the interactions to be included in the models, and to aid in deciding which collinear variables (VIF >5) to remove from the dataset.

The MLRs were performed using the AIC (Akaike Information Criteria) selection criteria with i) forward selection; ii) backward selection; and iii) forward and backward selection. For each model obtained, the residuals were checked for: i) normality; ii) homoscedasticity; iii) independence; and iv) if ‘x’ is fixed. Also, it was verified the existence of influential points. After, the models were tested using ANOVA (drop 1 variable; F), and residuals from each model were compared. The ‘best’ model was after applied to the independent data set used for validation in Method 1.

2.4. Multimetric index development: The RMAT – Rocky shore Macroinvertebrates Assessment Tool

The RMAT was designed considering the results from Method 1 (steps G. and H. in section 2.3.1.2) and Method 2. First, reference conditions (RC) were searched among the literature for each of the selected indices. When RC were not available in the literature, the maximum value was selected (corresponding to highest quality) obtainable by the metric, or calculated a mean value between the maximum and the 95th percentile values (obtained at present). Second, EQR values were calculated (as the ratio of the sample value and the RC, and ranging from 0-1) for each of the 144 samples. The EQR >1 (for samples with values higher than the RC) were truncated to 1. Third, the RMAT was calculated per sample as the mean value of the indices’ EQR values, and after was calculated and presented per sampling site.

In this step, different models were tested, in which different weightings were given to the indices included in RMAT (combination rule). The purpose was to select the RMAT model with results best matching to the MarMAT’, emphasizing the indices that showed best response to the disturbance (regarding their final scores).

The model with the most similar behaviour to MarMAT, by showing at sampling sites 1, 2 and 3 the mean EQR values closest to MarMAT EQR values, was selected for further validation.

2.5. Validation of the RMAT

The ‘best’ model (=RMAT) was applied to independent data, gathered from Buarcos during summer 2009 (using the same methodology as for the first dataset) at different sites (except site 1) along the disturbance gradient (Fig. 1C). The results were compared to the MarMAT EQR values calculated for that period, and compared to the previous response provided by the model (section 2.4).

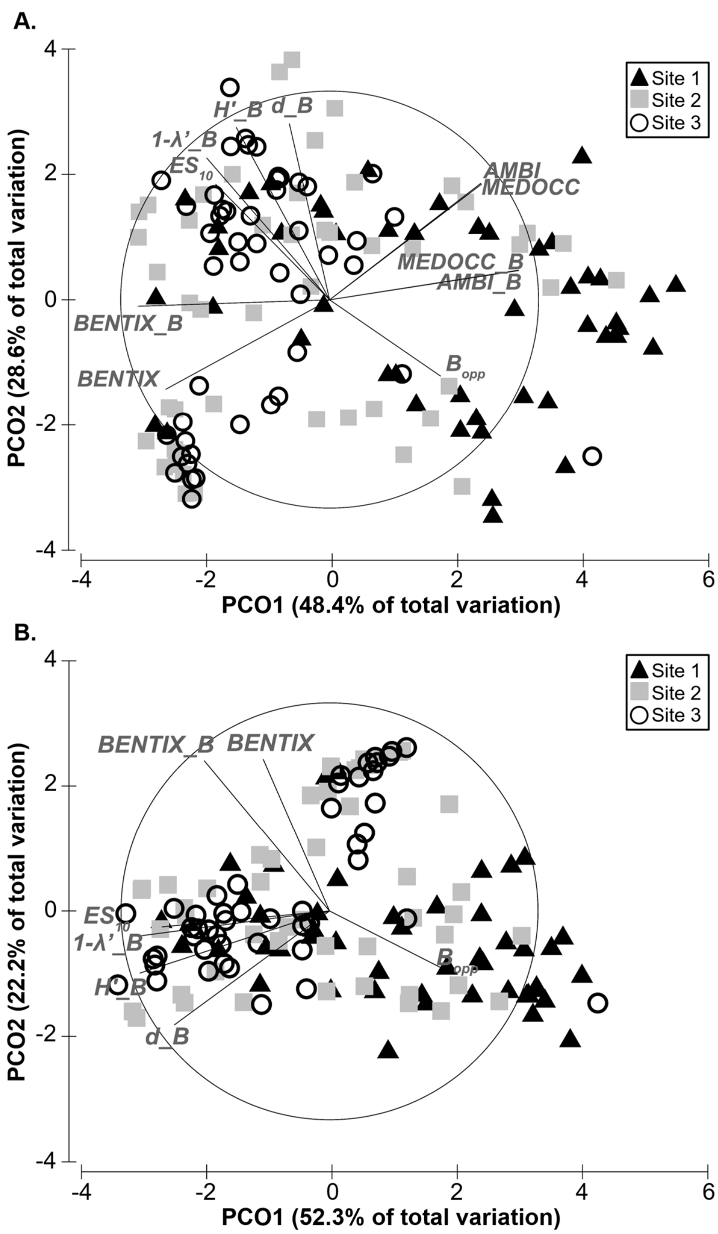

Figure 2. Principal coordinates (PCO) analysis plots considering eleven invertebrate indices (A.) and after removing high correlated (>0.9) indices (B).

3. Results

3.1. Method 1

3.1.1. Preliminary data set

The PCO ordination using the 11 macroinvertebrate indices (explaining 77.0% of total variability, 48.4% in PCO1) showed a separation between sites 1, 2 and 3 (Fig. 2A), mainly related to BENTIX_B, AMBI_B and MEDOCC_B, followed by BENTIX, MEDOCC and AMBI (PCA eigenvalues; Table 1A). Also, site 1 was more related to higher values of AMBI_B, MEDOCC_B, MEDOCC, AMBI and B_{opp}, while on the other hand site 3 was more related to higher values of BENTIX_B, BENTIX, H'_B, d_B, 1-λ'_B and ES_{10}.

Table 1. PCA eigenvalues (first two axes) for the eleven invertebrate indices (A.) and after removing high correlated (>0.9) indices (B.). Higher scores in bold.

		A.	PCA1	PCA2	B.	PCA1	PCA2
Biomass	Abundance/Diversity	d_B	0.084	-0.476	d_B	0.389	0.438
		ES_{10}	0.237	-0.312	ES_{10}	**0.421**	0.061
		H'_B	0.194	-0.466	H'_B	**0.476**	0.239
		1-λ'_B	0.255	-0.383	1-λ'_B	**0.472**	0.095
		B_{opp}	-0.231	0.206	B_{opp}	-0.310	0.242
	Taxonomic composition	AMBI_B	**-0.393**	-0.080			
		MEDOCC_B	**-0.393**	-0.079			
		BENTIX_B	**0.398**	0.017	BENTIX_B	0.314	**-0.580**
Density		AMBI	**-0.314**	-0.315			
		MEDOCC	**-0.316**	-0.313			
		BENTIX	**0.340**	0.242	BENTIX	0.166	**-0.586**

A. The ES_{10} showed significant differences between site 3 and the other two sites (PERMANOVA; Table 2). The remaining indices (except H'_B and d_B) showed differences between site 1 and the other two sites. The H'_B found differences between sites 1 and 3. For d_B the separation of sites was not statistically significant.

B. The RELATE routine showed significant relations between MarMAT and all macroinvertebrate indices except d_B (Table 3). Relationships were stronger (higher rho) between similarity matrices of MarMAT and BENTIX, followed by B_{opp}, MEDOCC, AMBI, BENTIX_B, and AMBI_B and MEDOCC_B.

C. Pearson correlations were very high between AMBI_B and MEDOCC_B, and AMBI and MEDOCC (~+1), followed by the comparisons of BENTIX_B with the prior two, and BENTIX with the latter two (~-1) (Table 4). The indices showing stronger correlations with MarMAT were AMBI_B (-0.487), MEDOCC_B (-0.487), BENTIX_B (+0.476), BENTIX (+0.472), AMBI (-0.463), MEDOCC (-0.460) and ES_{10} (+0.411).

Table 2. PERMANOVA results for the eleven macroinvertebrate indices. A. main tests. B. Pairwise tests (significant tests are presented).

A. Main tests			Pseudo-F	P(perm)
Biomass	Abundance/Diversity	d_B	2.357	0.0969
Biomass	Abundance/Diversity	ES_{10}	15.285	0.0001
Biomass	Abundance/Diversity	H'_B	8.320	0.0005
Biomass	Abundance/Diversity	1-λ'_B	9.073	0.0004
Biomass	Abundance/Diversity	B_{opp}	6.341	0.0014
Biomass	Taxonomic composition	AMBI_B	18.455	0.0001
Biomass	Taxonomic composition	MEDOCC_B	18.479	0.0001
Biomass	Taxonomic composition	BENTIX_B	19.058	0.0001
Density	Taxonomic composition	AMBI	10.706	0.0002
Density	Taxonomic composition	MEDOCC	10.730	0.0001
Density	Taxonomic composition	BENTIX	12.925	0.0001

B. Pairwise tests				t	P(perm)
Biomass	Abundance/Diversity	ES_{10}	Site 1, Site 3	5.254	0.0001
			Site 2, Site 3	3.748	0.0005
Biomass	Abundance/Diversity	H'_B	Site 1, Site 3	3.992	0.0002
Biomass	Abundance/Diversity	1-λ'_B	Site 1, Site 2	3.059	0.0026
			Site 1, Site 3	4.244	0.0001
Biomass	Abundance/Diversity	B_{opp}	Site 1, Site 2	2.590	0.0092
			Site 1, Site 3	3.008	0.0010
Biomass	Taxonomic composition	AMBI_B	Site 1, Site 2	4.291	0.0001
			Site 1, Site 3	6.151	0.0001
Biomass	Taxonomic composition	MEDOCC_B	Site 1, Site 2	4.308	0.0001
			Site 1, Site 3	6.143	0.0001
Biomass	Taxonomic composition	BENTIX_B	Site 1, Site 2	4.387	0.0001
			Site 1, Site 3	6.219	0.0001
Density	Taxonomic composition	AMBI	Site 1, Site 2	2.918	0.0050
			Site 1, Site 3	4.854	0.0001
Density	Taxonomic composition	MEDOCC	Site 1, Site 2	3.013	0.0049
			Site 1, Site 3	4.781	0.0001
Density	Taxonomic composition	BENTIX	Site 1, Site 2	3.296	0.0015
			Site 1, Site 3	5.102	0.0001

Table 3. RELATE relationships between MarMAT and each invertebrate index.

			Rho	p
Biomass	Abundance/Diversity	d_B	-0.018	0.7532
		ES_{10}	0.129	0.0001
		H'_B	0.047	0.0181
		1-λ'_B	0.161	0.0001
		B_{opp}	0.213	0.0001
	Taxonomic composition	AMBI_B	0.197	0.0001
		MEDOCC_B	0.197	0.0001
		BENTIX_B	0.205	0.0001
Density		AMBI	0.208	0.0001
		MEDOCC	0.209	0.0001
		BENTIX	0.261	0.0001

3.1.2. Final data set

The PCO (Fig. 2A) and PCA (Table 1A) analyses showed BENTIX_B distinguished sampling sites better than AMBI_B and MEDOCC_B. The BENTIX and BENTIX_B where the indices most similar to MarMAT (RELATE, Table 3). Considering this and previous findings from Vinagre et al. (2016b) where BENTIX performed better than AMBI and MEDOCC, and BENTIX_B performed better than AMBI_B and MEDOCC_B, in terms of showing high correlations (namely among these six indices) the AMBI_B, MEDOCC_B, AMBI and MEDOCC were removed from the data set previous to further analysis.

D. In the second PCO ordination (with seven indices, explaining 74.5% of total variability) separation of sites was again observed (Fig. 2B), with higher variation of data being explained by PCO1 (52.3%), which mainly related to H'_B, 1-λ'_B and ES_{10} (PCA eigenvalues; Table 1B). This was accompanied by less dispersion of the sites data across PCO2 and a slight rotation in the data cloud, in which could also be seen a slight separation of sites, mainly related to BENTIX and BENTIX_B.

Table 4. Pearson correlations between all indices. Highest correlations in bold.

	Macroinvertebrates										Macroalgae
	Biomass							Density			
	Abundance/Diversity				Taxonomic composition						
	ES_{10}	H'_B	1-λ'_B	B_{opp}	AMBI_B	MEDOCC_B	BENTIX_B	AMBI	MEDOCC	BENTIX	MarMAT
d_B	+0.542	+0.781	+0.775	-0.196	+0.043	+0.041	+0.062	+0.217	+0.207	-0.055	+0.128
ES_{10}		+0.789	+0.589	-0.303	-0.311	-0.310	+0.361	-0.191	-0.190	+0.283	+0.411
H'_B			+0.804	-0.447	-0.211	-0.212	+0.318	+0.063	+0.061	+0.071	+0.273
1-λ'_B				-0.442	-0.387	-0.388	+0.472	-0.108	-0.117	+0.228	+0.329
B_{opp}					+0.557	+0.558	-0.606	+0.013	+0.014	-0.061	-0.258
AMBI_B						**+1.000**	**-0.984**	+0.582	+0.584	-0.611	-0.487
MEDOCC_B							**-0.984**	+0.580	+0.583	-0.611	-0.487
BENTIX_B								-0.531	-0.536	+0.588	+0.476
AMBI									**+0.999**	**-0.965**	-0.463
MEDOCC										**-0.969**	-0.460
BENTIX											+0.472

E. The MarMAT showed increasing quality from site 1 < site 2 < site 3, as indicated by the increasing mean values in that direction. All indices presented a response parallel to MarMAT, with mean and median values increasing (decreasing in the case of B_{opp}) from site 1 to site 3 (Fig. 3). The indices showed lower variation (lower standard deviation and box and whiskers size) within site 3 (variation of Bopp, BENTIX, BENTIX_B and 1-λ'_B decreased from site 1 to site 3; variation of ES10 and H'_B was comparable across sites). The exceptions were the d_B showing lower variation within site 1, and the H´_B showing higher variation within site 3 (Fig. 3).

F. The multivariate SIMPER (Table 5A) calculated average squared distances (ASD) within sites which decrease from site 1 > site 2 > site 3, indicating larger data variation within site 1 contrary to site 3. Within site 1, the least contribution to the ASD was from the d_B, H'_B, ES_{10} and BENTIX_B; at site 2 these were the B_{opp}, ES_{10} and H'_B; and at site 3 they were the BENTIX, 1-λ'_B and BENTIX_B. The ASD was higher between sites 1 and 3, mainly due to the ES_{10}, B_{opp}, H'_B, BENTIX_B and BENTIX. Between sites 1 and 2 the top contributors to the ASD were the BENTIX, BENTIX_B, 1-λ'_B and B_{opp}. Between sites 2 and 3 the ASD was lower, which was related to the d_B, ES_{10} and H'_B.

In the univariate SIMPER (Table 5B), d_B showed the lowest ASD within site 1 and also between sites 1 and 3. The ES_{10} and H'_B, despite showing the highest ASD within site 3, also showed the highest ASD between sites 1 and 3. The 1-λ'_B, BENTIX_B and BENTIX showed the lowest ASD within site 3 and the highest between sites 1 and 2, followed by sites 1 and 3. The B_{opp} showed the best results, with higher ASD within site 1 and between sites 1 and 3.

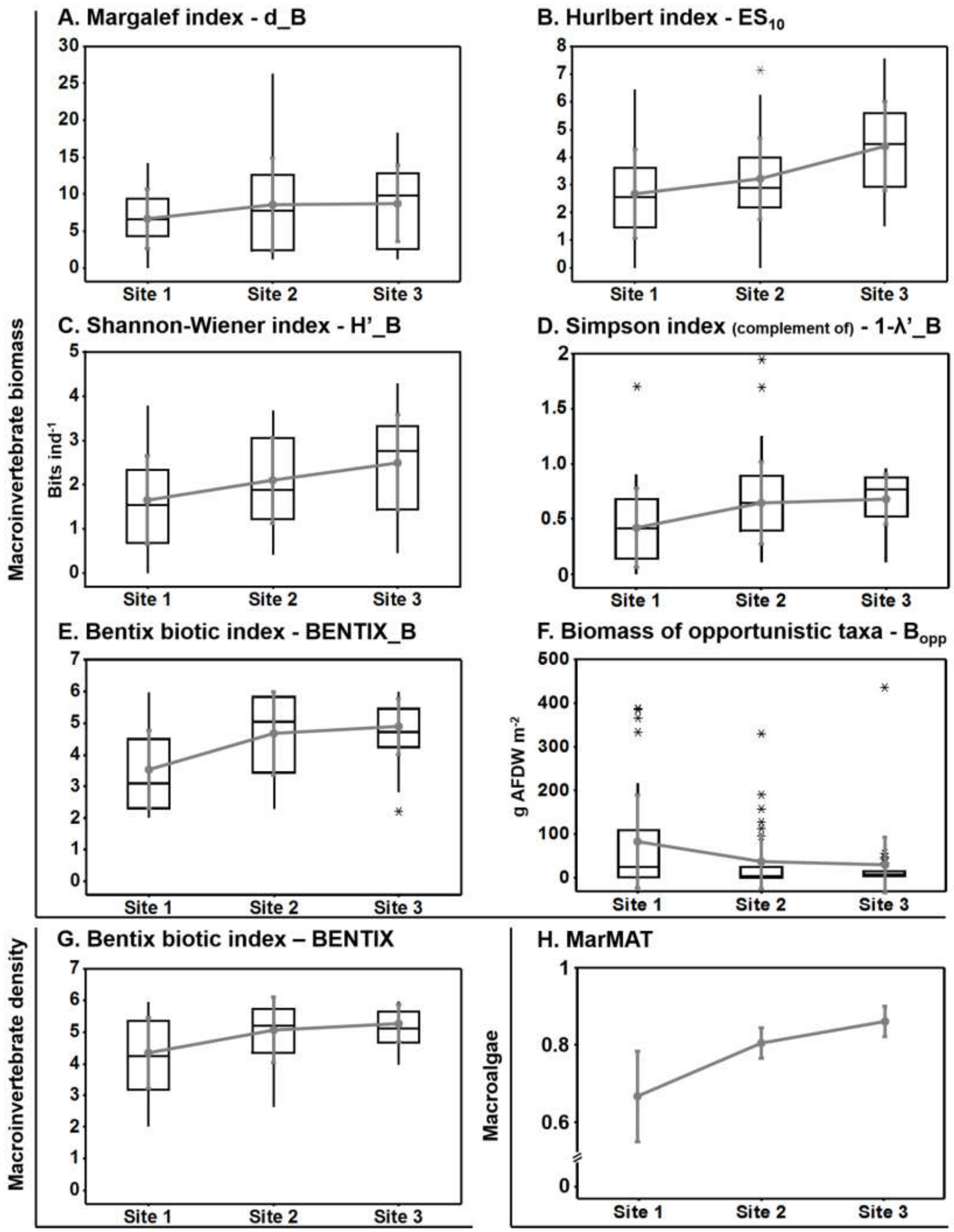

Figure 3. Comparison of invertebrate indices with MarMAT. Mean values (and standard deviation) in grey. Box plots in black – Box: mid line = 50th percentile (median); bottom and top of the box = 25th (Q1) and 75th (Q3) percentiles, respectively; lower and upper whiskers = [Q_1-1.5(Q_3-Q_1)] and [Q_3+1.5(Q_3-Q_1)], respectively; outliers = values outside whiskers limits (*).

Table 5. SIMPER analysis on factor 'Site'. A. Multivariate analysis, showing the indices contribution (%) to the average squared distances (in bold) within sites (shaded boxes) and between sites (non-shaded boxes). B. Univariate analysis, showing the distances within sites (shaded boxes) and between sites (non-shaded boxes).

A.

	Site 1		Site 2		Site 3	
Site 1	**7.18**	%				
	d_B	8.08				
	H'_B	12.15				
	ES_{10}	12.24				
	BENTIX_B	12.60				
	1-λ'_B	15.13				
	BENTIX	16.86				
	B_{opp}	22.94				
Site 2	**16.10**	%	**6.77**	%		
	BENTIX	16.76	B_{opp}	8.31		
	BENTIX_B	16.61	ES_{10}	10.84		
	1-λ'_B	16.50	H'_B	12.24		
	B_{opp}	15.36	BENTIX	15.18		
	d_B	12.91	BENTIX_B	15.26		
	H'_B	11.42	1-λ'_B	17.38		
	ES_{10}	10.44	d_B	20.79		
Site 3	**16.33**	%	**11.91**	%	**4.67**	%
	ES_{10}	16.82	d_B	19.45	BENTIX	7.14
	B_{opp}	15.94	ES_{10}	17.32	1-λ'_B	9.27
	H'_B	15.20	H'_B	16.40	BENTIX_B	9.80
	BENTIX_B	14.89	1-λ'_B	13.31	B_{opp}	12.46
	BENTIX	14.38	BENTIX_B	12.49	ES_{10}	19.01
	1-λ'_B	12.60	BENTIX	11.54	d_B	20.47
	d_B	10.17	B_{opp}	9.49	H'_B	21.86

B.

d_B	Site 1	Site 2	Site 3
Site 1	15.96		
Site 2	57.18	38.72	
Site 3	45.66	63.69	26.31

ES_{10}	Site 1	Site 2	Site 3
Site 1	2.49		
Site 2	4.76	2.08	
Site 3	7.78	5.85	2.52

H'_B	Site 1	Site 2	Site 3
Site 1	0.97		
Site 2	2.04	0.92	
Site 3	2.76	2.17	1.13

1-λ'_B	Site 1	Site 2	Site 3
Site 1	0.12		
Site 2	0.30	0.13	
Site 3	0.23	0.18	0.05

BENTIX_B	Site 1	Site 2	Site 3
Site 1	1.48		
Site 2	4.38	1.69	
Site 3	3.98	2.44	0.75

B_{opp}	Site 1	Site 2	Site 3
Site 1	11035.0		
Site 2	16569.1	3772.6	
Site 3	17441.8	7571.9	3901.9

BENTIX	Site 1	Site 2	Site 3
Site 1	1.19		
Site 2	2.66	1.01	
Site 3	2.32	1.36	0.33

G. The DistLM analysis between the MarMAT EQR values and the final seven indices highlighted the variables with most potential to be included in the final model (Table 6). Entering all variables in the model, all except d_B were statistically significant ($p<0.05$). The BENTIX_B (0.227), BENTIX (0.226) and ES_{10} (0.16) showed the highest contributions in the model. Using the backward selection criterion, all variables except those three were removed from the model. Using forward, step-wise and BEST selection criteria those three variables were too the ones selected (BIC = -709.7, R^2 = 0.3384) for the model.

Table 6. DistLM analyses showing invertebrate indices which explain most the MarMAT data, and the proportion of data explained (in grey).

A. Enter all variables

	Pseudo-F	p	Proportion
d_B	2.351	0.1282	0.0163
ES_{10}	28.780	0.0001	0.1685
H'_B	11.446	0.0010	0.0746
1-λ'_B	17.274	0.0001	0.1085
BENTIX_B	41.646	0.0001	0.2268
B_{opp}	10.148	0.0028	0.0667
BENTIX	40.779	0.0001	0.2231

BIC	R^2
-691.29	0.3452

B. Backward selection

Starting solution: All variables

BIC	R^2
-691.29	0.3452

	Pseudo-F	p	Proportion	Cumulative	BIC
-d_B	0.031	0.8585	0.0002	0.3451	-696.23
$-B_{opp}$	0.584	0.4372	0.0028	0.3423	-700.59
-1-λ'_B	0.601	0.4359	0.0029	0.3394	-704.93
-H'_B	0.204	0.6525	0.0010	0.3384	-709.69

Best solution: ES_{10}, BENTIX_B and BENTIX

BIC	R^2
-709.69	0.3384

C. Forward and D. Step-wise selection

	Pseudo-F	p	Proportion	Cumulative	BIC
+BENTIX_B	41.646	0.0001	0.2268	0.2268	-697.17
$+ES_{10}$	13.049	0.0005	0.0655	0.2923	-704.95
+BENTIX	9.766	0.0017	0.0461	0.3384	-709.69

Best solution: ES_{10}, BENTIX_B and BENTIX

BIC	R^2
-709.69	0.3384

E. BEST selection

Best solutions

Number of variables	BIC	R^2
1: BENTIX_B	-697.17	0.2268
2: ES_{10} and BENTIX	-707.87	0.3065
3: ES_{10}, BENTIX_B and BENTIX	-709.69	0.3384

H. Ultimately, the seven macroinvertebrate indices were scored considering their performance in each analysis (steps A.-C. in section 2.3.1.1, and steps D. and F. in section 2.3.1.2) (Table 7). The indices with highest final scores (sum of all scores), reflecting best overall performance, were the BENTIX_B, BENTIX and ES_{10}. Accordingly, these were the indices selected, together with those from step G. (section 2.3.1.2), to integrate the RMAT.

Table 7. Scores given to invertebrate indices in each step (A.-D. and F.) of Method 1 (highlighted in grey), and their final score (highlighted in black).

A. **PERMANOVA**

d_B	1
ES_{10}	3
H'_B	2
1-λ'_B	3
BENTIX_B	3
B_{opp}	3
BENTIX	3

B. **RELATE** (with MarMAT)

d_B	1
ES_{10}	3
H'_B	2
1-λ'_B	4
BENTIX_B	5
B_{opp}	6
BENTIX	7

C. **Correlations** (with MarMAT)

d_B	1
ES_{10}	5
H'_B	3
1-λ'_B	4
BENTIX_B	7
B_{opp}	2
BENTIX	6

D. **PCA**

	PCA1	PCA2	Mean*
d_B	4	5	4.33
ES_{10}	5	1	3.67
H'_B	7	3	5.67
1-λ'_B	6	2	4.67
BENTIX_B	3	6	4.00
B_{opp}	2	4	2.67
BENTIX	1	7	3.00

F.(i) **Multivariate SIMPER**

	Site 1	Site 2	Site 3	Mean	Site 1 Vs Site 3
d_B	7	1	2	3.33	1
ES_{10}	5	6	3	4.67	7
H'_B	6	5	1	4.00	5
1-λ'_B	3	2	6	3.67	2
BENTIX_B	4	3	5	4.00	4
B_{opp}	1	7	4	4.00	6
BENTIX	2	4	7	4.33	3

F.(ii) **Univariate SIMPER**

	Within sites	Site 1 Vs Site 3	Mean**
d_B	1	1	1.00
ES_{10}	1	3	2.33
H'_B	1	3	2.33
1-λ'_B	2	2	2.00
BENTIX_B	2	2	2.00
B_{opp}	2	3	2.67
BENTIX	3	2	2.33

Final Score	
d_B	12.67
ES_{10}	**28.67**
H'_B	24.00
1-λ'_B	23.33
BENTIX_B	**29.00**
B_{opp}	26.33
BENTIX	**28.67**

*calculated as [((2 x 'PCA1') + 'PCA2') / 3]

**calculated as [((2 x 'Site 1 Vs site 3') + 'Within sites') / 3]

3.2. Method 2

The successive tests for collinearity between macroinvertebrate indices showed very high variable inflation factors (VIFs), meaning high collinearity between variables. The metrics showing highest VIF were sequentially removed from the data set, namely the AMBI, MEDOCC, AMBI_B and MEDOCC_B.

The best model found (AIC = -730.04; R^2 = 0.3933) included the indices ES_{10}, BENTIX_B and BENTIX, and interactions between BENTIX_B and the other two indices.

The final model was given as:

Y1 ~ 1 + ES_{10} + BENTIX_B + BENTIX + (BENTIX_B:ES_{10}) + (BENTIX_B:BENTIX).

The respective equation was given as:

Y = 0.270249 (±0.102833) **+ [0.062444** (±0.021953) ***ES_{10}] + [0.093982** (±0.030617) ***BENTIX_B] + [0.055431** (±0.024125) ***BENTIX] - [0.011275** (±0.004623) ***BENTIX_B:ES_{10}] - [0.009355** (±0.006211) ***BENTIX_B:BENTIX]**.

Although the model fulfilled the assumptions for MLR models, influential points were observed, which could be contributing for the low R^2 (<0.5). The model could be improved with transformation of the indices data. However, this was not done owing to the intended purpose which was to identify the most efficient (in this case, most significant) metrics, these being the ES_{10}, BENTIX_B and BENTIX.

3.3. Index development: The RMAT – Rocky shore Macroinvertebrates Assessment Tool

From Method 1 and Method 2, the indices selected to combine in the RMAT were the ES_{10}, BENTIX_B and BENTIX. Reference conditions (RC) for these indices in rocky shores could not be found in the literature. Therefore, for BENTIX_B and BENTIX were used the maximum values obtainable by the metrics, i.e., RC = 6 for both. For ES_{10}, since the metric does not have an upper limit; the RC was calculated as the mean of the maximum value obtained (7.570) and the 95th percentile value (6.385). Hence, RC = 7 for ES_{10}.

All models tested showed parallels with MarMAT, with increasing mean EQR values from site 1 < site 2 <site 3 (Table 8A). The model deviating least from MarMAT was calculated as RMAT = [(ES_{10} + (2 x BENTIX_B) + (2 x BENTIX)) / 5]. As an alternative, if using only biomass data is advantageous (e.g., due to time constraints), the best model would be alt-RMAT = [ES_{10} + (2 x BENTIX_B)) / 3].

For the MLR model the same trend as above was found, with increasing values from site 1 < site 2 <site 3. The MLR model showed amongst all models the least deviation, and highest correlation, from MarMAT. However, it showed an over-estimation of site 1 mean EQR value with regard to the MarMAT values (Table 8A).

3.4 Validation of the RMAT

After being applied to the independent data set, RMAT and alt-RMAT results were concordant with the previous ones, both showing mean EQR values increasing from site 1 < site 2 < site 3, and deviating the least (after the MLR) from MarMAT EQR values (Table 8B).

For comparative reasons only, the MLR model was also applied to the independent data. As previously, despite presenting the desired trend, it showed over-estimation of mean EQR values for sites 1 and 2.

Table 8. Models tested using the indices ES_{10} (X), BENTIX_B (Y) and BENTIX (Z): A. Mean ecological quality ratio (EQR) values using the original data set; B. Mean EQR values using independent data for validation. In black: models selected for validation; in dark grey: models with three indices; in light grey: models with two indices; in white: multiple linear regression (MLR) model, and MarMAT assessment results [(EQR and associated ecological quality status (EQS)]. In bold: highest correlations with MarMAT.

		Three indices										Two indices			MLR	MarMAT	
A.		X+Y+Z /3	X+2Y+Z /4	X+2Y+2Z /5	2X+Y+Z /4	2X+2Y+Z /5	3X+Y+Z /5	3X+2Y+Z /6	2X+3Y+Z /6	3X+3Y+Z /7	1.5X+1.5Y+Z /4	X+Y /2	2X+Y /3	X+2Y /3	AIC = -730.04 R^2 = 0.3933	EQR	EQS
RMAT samples	Site 1	0.551	0.557	0.586	0.507	0.521	0.480	0.496	0.530	0.508	0.532	0.475	0.442	0.509	0.735	0.667	G
	Site 2	0.678	0.700	0.723	0.622	0.650	0.588	0.617	0.669	0.638	0.661	0.608	0.556	0.660	0.786	0.806	H
	Site 3	0.756	0.767	0.784	0.722	0.737	0.701	0.717	0.748	0.729	0.744	0.708	0.678	0.739	0.812	0.861	H
Deviation from MarMAT	Site 1	0.116	0.110	0.081	0.160	0.146	0.186	0.170	0.137	0.159	0.135	0.191	0.225	0.158	-0.068		
	Site 2	0.127	0.106	0.082	0.184	0.156	0.218	0.189	0.137	0.168	0.145	0.198	0.250	0.146	0.019		
	Site 3	0.105	0.094	0.077	0.139	0.124	0.160	0.144	0.114	0.132	0.117	0.153	0.183	0.122	0.050		
Correlation with MarMAT		**0.579**	0.569	0.576	0.554	0.566	0.528	0.550	0.564	0.558	0.572	0.535	0.505	**0.539**	**0.627**		
B.																	
Validation samples	Site 1	0.433	0.435	0.465	0.394	0.403	0.370	0.381	0.409	0.390	0.414	0.357	0.329	0.384	0.679	0.470	M
	Site 2	0.465	0.472	0.503	0.418	0.433	0.389	0.407	0.443	0.419	0.445	0.385	0.348	0.421	0.700	0.630	G
	Site 3	0.571	0.572	0.611	0.520	0.531	0.490	0.504	0.539	0.515	0.546	0.472	0.438	0.507	0.749	0.750	G
Deviation from MarMAT	Site 1	0.037	0.035	0.005	0.076	0.067	0.100	0.089	0.061	0.080	0.056	0.113	0.141	0.086	-0.209		
	Site 2	0.165	0.158	0.127	0.212	0.197	0.241	0.223	0.187	0.211	0.185	0.245	0.282	0.209	-0.070		
	Site 3	0.179	0.178	0.139	0.230	0.219	0.260	0.246	0.211	0.235	0.204	0.278	0.312	0.243	0.001		
Correlation with MarMAT		0.406	0.376	**0.409**	0.396	0.377	0.387	0.375	0.361	0.363	0.388	0.325	**0.334**	0.310	**0.397**		

4. Discussion

The WFD demands a sectoral approach for water quality assessments, undertaken individually for each water body and using each mandatory BQE. In the WFD context, macroalgae have been the biological quality element receiving most of the attention of researchers and monitoring teams in rocky shores. Quality assessment tools developed for rocky shores in the scope of WFD were mainly based on macroalgae, and only a few have explored macroinvertebrate communities alone, or their features, to produce any assessment tool, or part of it (Birk et al., 2012).

Taking the findings from Vinagre et al. (2016b) into account, the response of the 11 macroinvertebrate indices tested in the present work was not surprising. Using data from the summer, which was found as the best season (compared to winter) for monitoring activities, the indices could capture the disturbance gradients by showing increasing values (decreasing in the case of total biomass of opportunists – B_{opp}) from site 1 < site 2 < site 3. The different analyses performed within Method 1 and Method 2 allowed for the selection of the 'best' indices (i.e., better related to the disturbance gradients) to integrate the proposed multimetric index (RMAT). Therefore, the combination of the mean EQR values of the 'best' indices should also provide a response similar to the indices alone, which was confirmed, and afterwards validated using independent data. The best model found (i.e., showing EQR values increasing from site 1< site 2 < site 3, and deviating least from MarMAT) was RMAT = [(ES_{10} + (2 x BENTIX_B) + (2 x BENTIX)) / 5]. Within the RMAT, results from Bentix index seem duplicated. However, the metrics used are different since each (BENTIX and BENTIX_B) use different parameters (density and biomass, respectively) of the communities, which have shown to be not redundant, but complementary (Vinagre et al., 2016a). In fact, their combined use in another metric (W-statistic) did not show, by far, such good performance (Vinagre et al., 2016b). Also, other metrics (e.g., AMBI, multivariate AMBI – M-AMBI) have been tested with success, showing different results when using biomass or density data, or when applying different data transformation to those parameters, in soft-bottoms (Warwick et al., 2010; Muxika et al., 2012; Cai et al., 2014).

The proposed RMAT is compliant with WFD requirements, because: i) the selected indices cover the abundance and taxonomic composition parameters from rocky shore benthic macroinvertebrate communities and ii) the indices respond well to disturbance. The indices are easy to apply and their responses to disturbance, namely organic enrichment, are well known. This allows for a higher confidence when interpreting the results of assessments (Borja and Dauer, 2008); iii) the indices were used in combination, constituting a multimetric assessment tool; iv) the output of the RMAT is provided as an ecological quality ratio (EQR), varying from zero to one (0 to 1), as the deviation from the reference condition point; and v) the EQR scale is divisible by adjustable boundary thresholds into 5 quality classes (Bad, Poor, Moderate, Good, High) to report the EQS of the system. The RMAT has therefore shown potential to be applied to rocky shore benthic macroinvertebrates for quality assessments in the scope of the WFD.

As was previously suggested (Vinagre et al., 2016a, b, 2017), the rocky shores surveyed were under moderate (intermediate) disturbance. This was shown by the MarMAT which, considering each shore, presented EQR values covering only from moderate to high status. A similar trend was also shown by the RMAT (mean EQR at site level). However, several individual samples showed a low RMAT value, an EQR close to 0 (zero), which means the multimetric index should be able to report to all of the five quality classes. Hence, the RMAT should be able to show a low EQS (Bad status) when disturbance is significant. The RMAT will need further validation (and setting of boundaries between all quality classes), testing on different rocky shores with greater disturbance, a crucial step in refining the accuracy and precision of a developing index (Borja et al., 2009b). In the WFD context, there is also the need to calibrate its use across the long-known geographic gradients in assemblage composition on European rocky shores (e.g., Southward et al, 1995).

Concerning other marine Directives and Regional Conventions in Europe (e.g., MSFD, OSPAR, HELCOM), even though the use of rocky shore macroinvertebrate indicators could be effective and easy to use, environmental assessment approaches rarely have seen that possibility implemented. For example, in the MSFD list of Descriptors (European Commission, 2010) most of the indicators are based on water column and sedimentary habitat organisms; those from communities of rocky shores not frequently considered. It is true the former habitats have greater extent in European Shelf Seas, but the development of indicators based on hard substratum, able to respond to anthropogenic disturbance, may be of importance for several of the above-mentioned Directives (van Hoey et al., 2010; Rice et al., 2012). The use of more complex macroinvertebrate indicators has not occurred, then, RMAT is a valid proposal to integrate the assessment for MSFD Descriptors (e.g., D1 – Biological Diversity and D6 – Seafloor Integrity). Here, the RMAT can be considered as a promising indicator, contributing to improve the holistic perspective of the study area. With the RMAT, instead of using basic measures from benthic rocky shore community, a more complex type of indicator, combining different perspectives of the community, will be available.

In parallel to the RMAT, a second best alternative model was determined, including only indices calculated using biomass: alt-RMAT = [(ES_{10} + (2 x BENTIX_B)) / 3]. Although being not as accurate as the RMAT, the alt-RMAT could be helpful when time is a constraint, since there is no need to count every organism. For a quick analysis (rough estimations per sample) were taken Vinagre et al. (2016b) summer and winter data as an example: i) sample processing: 2.5 h (includes removing all formalin solution used for preservation, removing any debris such as empty shells hardening the sorting, checking macroalgae and debris for organisms, sort all organisms); ii) counting all individual organisms: summer data – 1.8 h (636.3 mean organisms per sample, multiplied by 10 sec to count each organism), and winter data – 1.6 h (were counted 561.8 mean organisms) (includes preparing/using equipment, counting the organisms, and preparing the data matrix); iii) estimating biomass: the organisms of each species from each sample were separately placed in containers, dried and weighed, burnt and re-weighed (biomass was calculated as ash-free dry weight). Summer data – 0.15 h (mean 27.7 containers per sample, multiplied by 10 sec for processing each container), and winter data: 0.13 h (mean 23.9 containers) (includes preparing/using equipment, weighing twice each container, and preparing the data matrix). This is disregarding the time needed to dry (at least 72 h) and burn (8 h) the organisms. The time spent counting all organisms would correspond to about 40% and 37% for summer and winter data, respectively, of sample processing, whilst to calculate their biomass would constitute only 3.5% and 3.2%. The most difficult task in the whole process is taxonomy, and would require a minimum 50% of the total processing time of a sample. Therefore, considering all estimates, if time is a constraint, using alt-RMAT instead of RMAT could save between 18.5-20% of processing time per sample.

5. Conclusions

The present work addressed a gap in the implementation of the WFD, where no tool was available to exclusively assess macroinvertebrates on rocky shores. The RMAT is a multimetric index compliant with the WFD, integrates widely known ecological indices (Hurlbert and Bentix indices) which shown, during summer season, best performance in distinguishing sites along disturbance gradients (moderate organic enrichment).

As it has shown potential to be used in quality assessments in the scope of the WFD and other statutory requirements, its applicability needs further validation, using data covering other geographical areas and stronger environmental degradation, to adequately define the five EQS classes and adjust boundaries between them.

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