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Improved Total–Species Accumulation Curve for Reliable Estimation of Regional Species Richness: An Application to Macroalgae Diversity on Bioconstructions from the Northern Adriatic Sea (Mediterranean Sea)

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Abstract

Traditional species richness estimators often assume spatial homogeneity in species distribution, which can lead to underestimating biodiversity, especially in large, ecologically complex areas. The Total–Species (T–S) curve may provide an accurate framework for estimating γ-diversity by accounting for compositional variation across spatial subunits. Our study tested the T–S curve model, modified to account for species rarity and patterns of β-diversity, to estimate macroalgal richness in the northeast Adriatic (Mediterranean Sea), an area where the total macroalgal diversity is known and a comprehensive reference list is available (487 species). Uncertainty in species richness estimates from T–S curves was quantified as 95%CI based on bootstrapping, and a sensitivity analysis was also carried out to quantify changes in estimates under different settings. Other parametric and non-parametric estimators, including the classic T–S curve, largely under- or overestimated the total species richness if compared to the refined T–S model, which returned a realistic estimate of 393 species in total. Our results demonstrate that the T–S curve modified to consider species rarity, and refined for potential biases associated with erroneous quantification of small-scale patchiness and spatial variations in assemblage composition, allowed for more realistic extrapolations of γ-diversity over large areas.

Keywords: species-accumulation curves; species richness estimators; patchiness; β-diversity; γ-diversity; marine biodiversity

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1. Introduction

Conventional approaches for estimating species richness typically overlook spatial variation in biodiversity across the study area, assuming high spatial homogeneity in species distribution [1]. However, this assumption can be violated when the aim is to estimate species richness in large areas (i.e., γ-diversity), or when the investigated area is characterized by a complex mosaic of habitats [2–4], potentially leading to an underestimate of the actual γ-diversity [1,2,5–7].

Nonparametric estimators [8] incorporate spatial heterogeneity and are commonly used to obtain more accurate estimates of the actual number of species in a community under reduced sampling effort. Yet they remain affected by changes in species-abundance distributions [9], high levels of spatial heterogeneity in species composition [10] and, more importantly, these estimators are mainly intended to yield lower-bound estimates at local scales [1,11,12]. Comparable limitations affect asymptotic models (e.g., Michaelis–Menten function), which fit smoothed sample-based accumulation curves that often underestimate species richness in large areas [13,14]. Other approaches, such as the application of mixture models [6] and non-asymptotic curves, have noticeable potential, although they poorly perform, respectively, in estimating γ-diversity over regions > 10 times larger than the sampled surface and in accounting for the heterogeneity in species distribution over the investigated region [15,16].

The total–species (T–S) accumulation curve proposed by Ugland et al. [4] models species richness at an increasing number of samples by fitting a semi-log function to the terminal points of a collection of accumulation curves from different subunits within the whole area of interest. This allows for incorporating both compositional variation among samples and the spatial heterogeneity in species composition among subunits (i.e., β-diversity), producing realistic estimates of γ-diversity with respect to classic estimators [2,3,17]. However, the T–S curve can be sensitive to species rarity [3], and/or to the mismatch between the selected spatial units used in the accumulation model and the actual variations in β-diversity within the total area [2], leading to overestimating species richness [17].

Bevilacqua et al. [18] modified the T–S curve approach, introducing an objective identification of spatial subunits based on the analysis of β-diversity patterns within the total area of interest, and to account for small-scale patchiness and species rarity. Specifically, the new framework was developed to reduce potential biases in species richness estimates from the T–S curve due to (i) mis-specified spatial subunits that fail to capture true β-diversity patterns in the area of interest, (ii) small-scale patchiness (i.e., mis-assignment of α-diversity to β-diversity), and (iii) differential saturation rates between common/intermediate species versus rare species. This modified approach to the T–S curve was successfully applied to mollusk assemblages from coastal areas in the south Adriatic Sea (Mediterranean Sea), providing more accurate estimates of family richness if compared to classic estimators, approximating the true total number of families known in the region [18]. However, although the coefficients of the T–S curve can be strongly correlated at different taxonomic levels (generally until the level of family), this indicates that the improvements to the T–S curve could also lead to improved estimates at the species level [18], although decisive tests still lack.

The main issue when assessing the effectiveness of species richness estimators consists of the general lack of case studies where the total species richness in the region of interest is known. In fact, if nonparametric estimators may lead to reliable lower-bound estimates of species richness [19], controlling for overestimation is more complicated in the absence of an exhaustive record of species in a given area that could serve as a reference. In this respect, macroalgae diversity in the northeast Adriatic Sea can be one of the rare exceptions where the full list of species is virtually known. Macroalgal diversity in this region has been extensively studied for two centuries, and a recent work published the full list of macroalgae in the region [20], providing the opportunity to test the performance of the modified T–S curve in estimating γ-diversity. The list was obtained from an extensive review of scientific papers, gray literature, archival records and floristic data collected in the area spanning over the last two centuries to build an exhaustive, updated taxonomic dataset of macroalgae on hard substrates (including natural rocky reefs, artificial reefs, and bioconstructions) in the northeast Adriatic region [20].

Based on this reference of regional species richness and a large dataset of macroalgae sampled on coralligenous bioconstructions in the region, the present study aims to (i) test the performance of the classic T–S curve as proposed by Ugland et al. [4] and further developed by Bevilacqua et al. [18] in providing reliable estimates of macroalgal species richness in this biogenic habitat over the study region, and to (ii) compare estimates from the modified T–S curve with estimates from the application of classic parametric and nonparametric species richness estimators.

2. Materials and Methods

2.1. Study Area, Dataset, and Species Reference List

The northern Adriatic Sea, the shallowest and northernmost basin of the Mediterranean Sea, is largely characterized by mobile sandy–silty sediments, but hosts numerous coralligenous outcrops typical of the region spanning over the Italian and the Slovenian coastal shelves [21,22]. These structures, distributed between 3 and 13 nautical miles from the coast and at depths of 9–40 m, are highly variable in size (from ~1 m^2 blocks to several thousand m^2) and rarely exceed 4 m in height [21]. They provide hard substrates supporting mesophotic biogenic reef assemblages dominated by encrusting coralline algae (e.g., *Lithophyllum*, *Lithothamnion*, *Mesophyllum*, *Peyssonnelia* spp.) [23–25], which act as bioengineers and enhance habitat complexity. These reefs host remarkable biodiversity, with more than 170 macroalgal taxa and over 700 macroinvertebrate taxa recorded [23,26–28].

The dataset used to estimate the overall species richness of macroalgae on outcrops in this area was obtained from the works of Curiel et al. [27] and Falace et al. [23], which gathered and analyzed samples from several monitoring programs carried out between 2009 and 2011 (Table S1 in Supplementary Material).

Sampling strategies were homogeneous across all studies and sampling sites. Samples were collected by randomly sampling different outcrops from 4 zones representative of the distribution of bioconstructions in the region, based on combined environmental characteristics of geographic position, depth range, and distance from the coast [23,27]. In the dataset, each sample consisted of 3 replicate quadrats of 0.5 × 0.5 m randomly collected on the bioconstruction in different sampling sites (Figure 1, Supplementary Materials S1), for a total of 37 samples. In each replicate quadrat, the whole benthic assemblage was scraped off the substrate, and macroalgae were sorted and identified at the species level by expert algologists.

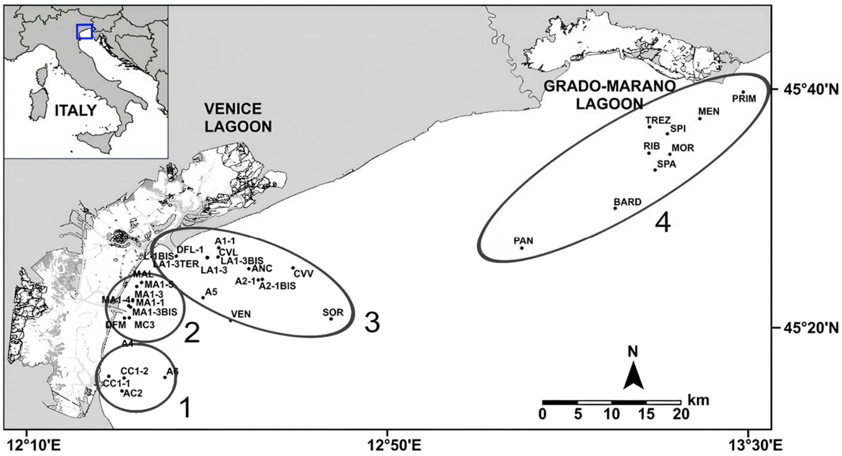

Figure 1. Study area modified from [27]. Points on the map corresponded to the 37 sampled outcrops in the 4 investigated zones identified by the numbered clusters (1–4). The blue square in the upper left corner indicate the larger geographic context of the study (North Adriatic Sea, Mediterranean Sea).

The species list of macroalgae provided by Falace et al. [20] (available at https://doi.org/10.1016/j.marenvres.2024.106828) was used as a reference to compare against species richness estimates in the region. In the list, a total of 487 species were reported, and this value was referred to as the upper bound macroalgal γ-diversity that can be found inhabiting bioconstructions in the study region (S_{Tot}^{Real}). The list encompasses all species found on hard substrates (including natural rocky reefs, artificial reefs, and bioconstructions) in the northeast Adriatic region, including ephemeral drift species (e.g., some species belonging to the genera *Cladophora* and *Ulva*) and some free-living coralline algae (i.e., *Lithophyllum racemus* (Lamarck) Foslie and *Phymatolithon calcareum* (Pallas) W.H.Adey & D.L.McKibbin ex Woelkerling & L.M.Irvine), which can nevertheless be associated with the outcrops.

2.2. The Classic Total–Species Curve

Structuring the Total–Species (T–S) curve involves generating a sequence of species accumulation curves (i.e., randomized accumulation of the number of species at increasing number of samples) from the random combinations of 1, 2, 3, . . . , n spatial units (i.e., subareas) within the study region (in this case, 100 randomizations of samples for each combination of subareas were used). Finally, the T–S curve is constructed by fitting a semi-logarithmic function to the terminal points of the nested accumulation curves (see Bevilacqua et al. [18] and Ugland et al. [4] for methodological details).

The intercept ($\hat{\mu}_S$) and the slope coefficient ($\hat{\beta}_S$) in the model are obtained by ordinary least squares regression. The estimated total species richness $\left(\hat{S}_{Tot}\right)$ in the whole area is calculated as follows (where $N = A/a$, with a being the area of one sample, and A the total area of interest, Equation (1):

$$\hat{S}_{Tot} = \hat{\mu}_S + \hat{\beta}_S \left(\ln N\right) \tag{1}$$

In this study, the area of the sample was $a = 0.75\ \mathrm{m}^2$ (i.e., the cumulative surface of the 3 replicate quadrats collected in each sampling site), whereas the total area of interest $A = 1200 \times 10^3\ \mathrm{m}^2$ was the surface of outcrops across all the investigated zones; it follows that $N = 1600 \times 10^3$ and $\ln(N) = 14.28$. The total surface of outcrops was estimated based on previous studies of habitat mapping integrating multibeam echosounder and side scan sonar surveys in the region [29].

As the T–S curve does not provide measures of dispersion for estimates, we applied a bootstrap procedure to generate 95% bias (z-score) corrected confidence intervals (CI). This resampling approach is commonly used to evaluate the variability of T–S curve estimates [3] as well as of other richness estimators (e.g., Eren et al. [30]). Bootstrapping was performed by data resampling with replacement for each combination of 1, 2, 3, . . . , n subareas. A total of 1000 bootstrap estimates were obtained, on which the 95%CI was constructed (the procedure was also carried out with 10,000 bootstrap estimates, leading to comparable 95%CI, see Table S1 in Supplementary Material).

Sensitivity analysis was performed to check for the potential effects of variations in the total area of interest A on species richness estimates. The analysis was performed by estimating $\hat{S}_{Tot}$ considering variations of ±10–20% in the total area A.

2.3. Modified T–S Curve Model

The modified T–S curve was implemented following the framework proposed by Bevilacqua et al. [18]. Firstly, to assess whether samples adequately captured local-scale (α) diversity (i.e., if the size of the sampled surface is effective in quantifying the α-diversity), we applied the method from Anderson & Santana-Garcon [31] involving the use of a pseudo-multivariate dissimilarity-based standard error to determine the appropriateness of sample size. This step allows checking if sample pooling should be applied to avoid the mis-assignment of α-diversity to β-diversity [32], with the ensuing overestimation of γ-diversity. Sample pooling was not considered necessary as a relatively low pseudo-multivariate standard error (MultiSE = 0.15) was characterized for the multivariate dataset. Also, since each sample in the dataset is the sum of 3 replicate subsamples collected on outcrops from distinct sites, 100 s to 1000 s m apart, this residual variability highlights an intrinsic property of the investigated assemblages rather than the effect of limited sample size.

The second step was to test if the subareas were able to represent the actual pattern of spatial heterogeneity in species composition among the macroalgal assemblages characterizing the outcrops within the study region. As accumulation curves assume virtual homogeneity in species distribution in the area of interest, a careful quantification of β-diversity patterns is crucial to identify distinct spatial subunits for the construction of the nested accumulation curves for the T–S model. In this study, the 4 zones (Figure 1) were identified as distinct spatial units for representative monitoring campaigns due to different locations, depths, and distances from the coast. However, while the outcrops within zones 1 and 2 were quite homogenous, with flat bioconstructions dominated by turf and reef-building algae respectively, both zones 3 and 4 included distinct portions of their sea bottom characterized either by flat or higher-elevation outcrops with different algae assemblage structures [23]. Therefore, changes in β-diversity among types of outcrops within zones can also be relevant, and portions of the seafloor with different types of outcrops in each zone, rather than zones alone, may be the appropriate spatial units to consider in the T–S accumulation curve. This hypothesis was tested through a permutational multivariate analysis of dispersion (PERMDISP) [33], to assess if both the variation in species composition within and among zones was significant, hence requiring a refinement of the original 4 spatial units. The dissimilarity matrix among samples was based on Jaccard's distance, and PERMDISP tests were performed with 9999 permutations. The analysis included 2 crossed factors, namely Zone (Z, i.e., the original subareas, 4 levels, fixed) and Type (T, i.e., flat and high-elevation outcrops, 2 levels, fixed). We anticipate that the analysis detected a significant Z × T interaction (pseudo-$F_{5,31}$ = 4.205, $p < 0.05$), indicating that both factors were relevant. Therefore, partitioning samples by the interaction factor could provide a more reliable representation of the β-diversity patterns than the clustering of samples based on factor "Zone" alone. This led to identify 6 instead of the original 4 spatial units, since both zones 3 and 4 were split into two new units (namely, 3a-3b and 4a-4b) characterized by the presence of different outcrop types (i.e., flat and higher elevation outcrops). The T–S curve (and 95%CI) was calculated based on 4 (i.e., the original zones) and 6 (the new spatial units) subareas to compare the relative estimates of species richness.

Finally, the T–S curve was modified to consider patterns of rarity among species. The 187 species in the dataset were divided, following Reichert et al. [3] and Bevilacqua et al. [16] and references therein, into common (species present in >10% of samples), intermediate (species present in 5–10% of samples), and rare (present in <5% of samples) [3,34,35]. The number of species $\hat{S}_{Tot}$ in the total area (±95%CI) was estimated as follows:

$$\hat{S}_{Tot} = S_{Obs}^{Com} + S_{Obs}^{Interm} + \hat{S}_{Tot}^{Rare} \qquad (2)$$

where S_{Obs}^{Com} and S_{Obs}^{Interm} were, respectively, the observed number of common and intermediate species in the area from randomized accumulation curves at saturation, whereas $\hat{S}_{Tot}^{Rare}$ was the estimated total number of rare species obtained from the T–S curve (Equation (1)) and based on the 6 subareas.

The analysis was run considering different percentages of occurrence to check for the sensitivity of estimates to varying rarity ranges. Specifically, the analysis was carried out considering a reduced (1–3% occurrence for rare species, 3–8% for intermediate species, and >8% for common species) and an increased (1–7% for rare species, 7–12% for intermediate species, and >12% for common species) range for rarity. All analyses were performed using R Studio 4.4.1 [36], package "vegan" [37] and the Primer-E software version 7 [38,39].

2.4. Comparison with Other Species Richness Estimators

Estimates from the modified T–S curve were also compared with those obtained from a set of species richness estimators, to evaluate the performance and reliability of the T–S curve with respect to other common-use estimators. We selected a set of species richness estimators representative of different approaches, including nonparametric estimators like the first-order Jackknife estimator (Jackknife 1), the Chao estimator for incidence data (Chao 2), and the Incidence-Based Cover Estimator (ICE), and asymptotic (modified exponential, Michaelis-Menten) and non-asymptotic parametric (logarithmic, power) functions. For parametric estimators, the goodness-of-fit was checked using the Wald-Wolfowitz runs test and the Corrected Akaike Information Criterion.

3. Results

3.1. Estimate of Total Species Richness from the Classic T–S Curve

The slope coefficient of the classic T–S curve based on the original 4 subareas (Figure S1 in Supplementary Material) was $\hat{\beta}_S = 55.99 \pm 1.23$ and the intercept $\hat{\mu}_S = -14.145 \pm 3.48$ ($p < 0.001$, $R^2 = 0.998$). The total number of species in the total area (Equation (1)) was estimated as $\hat{S}_{Tot} = 786$ (±95%CI: 720, 803). As expected, this value clearly overestimated (+61.39%) the maximum possible number of 487 species as a consequence of the mismatch between the 4 subareas and the actual β-diversity patterns in the total area (Figure 2).

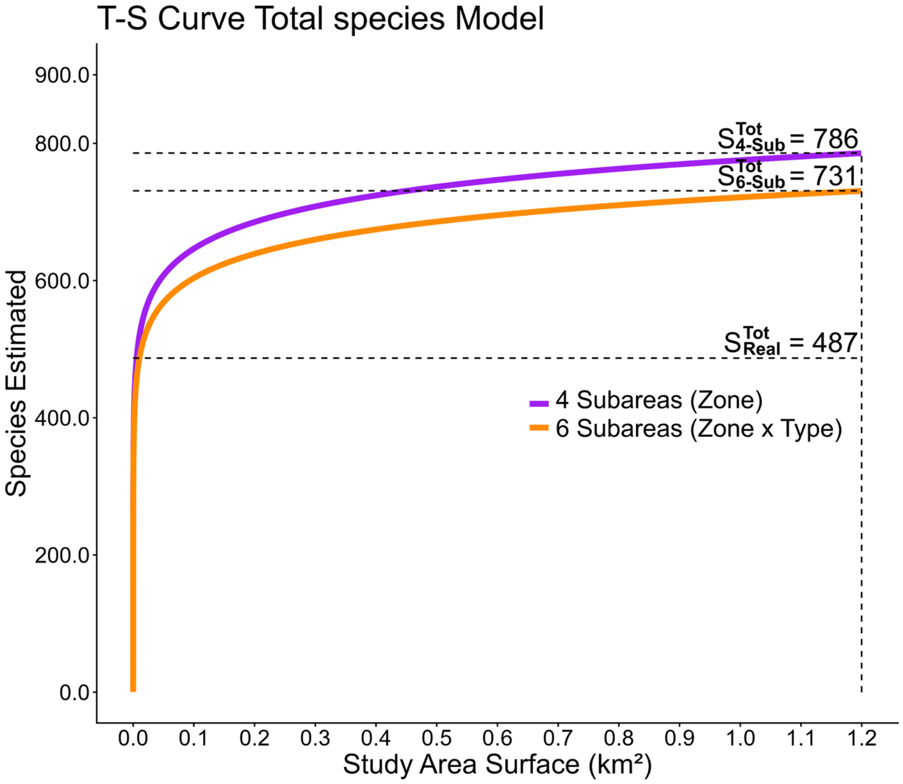

Figure 2. T–S curve based on the original 4 subareas and the 6 subareas. The estimates of total species richness based on the original 4 subareas ($\hat{S}^{Tot}_{4\,-\,Sub}$, purple line) and the refined 6 subareas ($\hat{S}^{Tot}_{6\,-\,Sub}$, orange line), and the threshold of the maximum species richness known for the area were also reported (S^{Real}_{Tot}).

3.2. Estimate of Total Species Richness from the Modified T–S Curve

The analysis of small-scale patchiness excluded the presence of undefined and 100%-dissimilarity values among samples and highlighted a normal distribution of pair-wise dissimilarities among samples (Figures S2 and S3 in Supplementary Material), indicating an adequate sample surface to characterize local-scale diversity of macroalgae and no need for sample pooling.

PERMDISP analysis detected a significant Z × T interaction (Table 1), indicating that the heterogeneity in species composition of macroalgae assemblages significantly varied across zones and types of outcrops. This result outlined that β-diversity among samples changed depending on both geographic location and type of outcrops, so that the 6 subareas (i.e., subarea 1, 2, 3a, 3b, 4a, 4b) are better aligned with the actual spatial patterns of β-diversity of macroalgae in the study area.

Table 1. PERMDISP results. The analysis was carried out among samples from different Zones and Type of habitats. dfn = degrees of freedom of the numerator; dfd = degrees of freedom of the denominator.

Source of Variation	**Dfn**	**Dfd**	***F***	***P* (Perm)**
Zone = Z	3	33	4.916	0.027
Type = T	1	35	0.121	0.758
$Z \times T$	5	31	4.205	0.047

The slope coefficient of the T–S curve based on the 6 subareas (Figure S4 in Supplementary Material) was $\hat{\beta}_S = 51.13 \pm 0.76$ and the intercept $\hat{\mu}_S = -2.94 \pm 2.12$ ($p < 0.001$, $R^2 = 0.999$). In this case, the T–S curve estimated a total number of species equal to $\hat{S}_{Tot} = 731$ (±95%CI: 744, 677), exceeding the full number of species in the area by +50.10% (Figure 2), but leading to reduce the overestimation with respect to the model based on the original 4 subareas of approximately 11%.

The randomized accumulation curves for common and intermediate species reached saturation after 27 and 35 samples, respectively (Figure 3), indicating that sample size ($n = 37$) was sufficient to detect all common ($S_{Obs}^{Com} = 73$) and intermediate ($S_{Obs}^{Interm} = 56$) species. The estimated parameters of the T–S curve (6 subareas) for rare species ($\hat{\beta}_S = 18.80 \pm 2.42$ and $\hat{\mu}_S = -15.82 \pm 6.73$; $p < 0.01$; $R^2 = 0.919$) resulted in 255 rare species, leading to an overall estimate of 382 (±95%CI: 389, 358) species, which represented a more realistic estimate falling below the maximum threshold of known total γ-diversity in the study area.

The sensitivity analysis revealed that applying different rarity thresholds (5–10%, 3–8%, and 7–12%) resulted in limited variations in species estimates (Table 2). The 5–10% rarity threshold and the 3–8% yielded the same estimate of species (381.9), while the 7–12% produced higher estimates (531.8). Similarly, modifying the surface area of the study region by adding or subtracting 10% or 20% lead to only marginal changes in species estimates.

Table 2. Sensitivity analysis for species richness estimates from T–S curve (6 subareas) at different rarity thresholds (i.e., considering as rare those species occurring in <5%, <3%, and <7% of samples) and variations in the total surface area *A* (±10%, ±20%).

	A	***A* + 10%**	***A* − 10%**	***A* + 20%**	***A* − 20%**
$\hat{S}_{Tot}$	731	735	725	740	719
$\hat{S}_{Tot}^{Rare}$ (<5%)	382	384	380	385	38
$\hat{S}_{Tot}^{Rare}$ (<3%)	382	384	380	385	378
$\hat{S}_{Tot}^{Rare}$ (<7%)	532	535	528	540	524

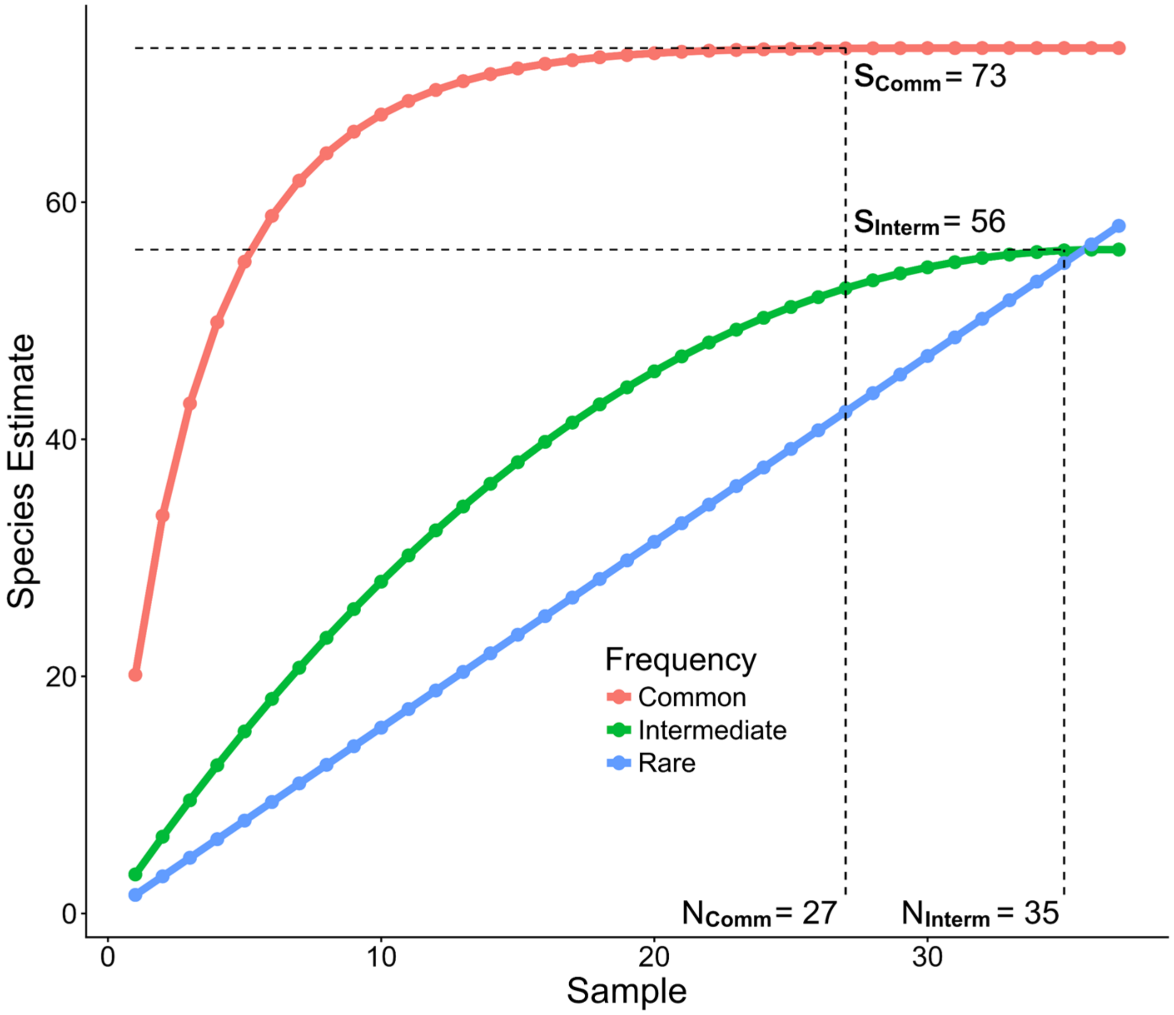

Figure 3. Randomized accumulation curves for common (red) and intermediate (green) species, and T–S curve (6 subareas) for rare species (blue). The number of common (S_{Obs}^{Com}) and intermediate (S_{Obs}^{Interm}) species at saturation and the number of samples required for saturation (N_{Com} = 27; N_{Interm} = 35) were also indicated.

3.3. Comparison with Other Species Richness Estimators

Species richness estimates varied widely across methods (Table 3). The modified T–S curve accounting for species rarity and based on the 6 subareas, was the only estimator providing a realistic estimate of total species richness, below the maximum threshold and, at same time, plausible considering the extension of the total area of interest. Logarithmic and power models largely overestimated the known biodiversity of 487 species. Conversely, the Jackknife 1, ICE, Chao 2 estimators, along with the modified exponential and Michaelis-Menten functions provided estimates very close to the total number of observed species in the samples (i.e., 187 species). Among parametric models, the Michaelis–Menten function showed the best fit based on the Corrected Akaike Information Criterion (AICc = 89.33), although the fit to data was unlikely for all models (runs test $p < 0.000$, in all cases) (see Table S2 in Supplementary Material).

Table 3. Species richness estimates (±95%CI) from the modified T–S curve and other parametric and nonparametric estimators. S_{Obs} is the total number of observed species in the samples; n is the number of samples; N is the number of samples of surface a needed to cover the total area of interest A; q_1 and q_2 are the number of singletons (species occurring only in one sample; in this study $q_1 = 58$) and double tons (species occurring only in two samples; in this study $q_2 = 46$), respectively; S_{Freq} and S_{Infr} are, respectively, the number of frequent (found in >10 samples) and infrequent (found in ≤10 samples) species in a sample, and γ^2_{ICE} is the coefficient of variation in the ICE; b and c are the coefficients of the parametric estimators. See [1] for further details.

Estimator	Formula	Estimate	95%CI
Modified T–S curve (6 subareas and rarity)	Equation (2)	382	(358, 389)
Jackknife 1	$S_{Obs} + q_1\left(\frac{n-1}{n}\right)$	245	(217, 230)
ICE	$S_{Freq} + \frac{S_{Infr}}{1-\frac{q_1}{S_{Infr}}} + \frac{q_1}{1-\frac{q_1}{S_{Infr}}}\gamma^2_{ICE}$	210	(196, 220)
Chao 2	$S_{Obs} + \frac{q_1^2}{2q_2}$	224	(217, 230)
Modified exponential	$ce^{b/N}$	201	(192, 210)
Michaelis–Menten	$\frac{cN}{b+N}$	238	(232, 244)
Logarithmic	$c + b\ln(N)$	706	(681, 731)
Power	cN^b	21,307	(17,729, 24,955)

4. Discussion

Marine community assembly is often the result of a complex interplay among environmental factors and biological processes, along with historical and biogeographic constraints [40–42], which shape the spatial distribution of species generating spatial variations in community composition from one place to another [43]. β-diversity, the primary descriptor of such spatial variations, quantifies changes in species composition among spatial units within the landscape [44], and ultimately, how local- and regional-scale diversity are related [45]. As the rate of species accumulation at increasing sampled area (or number of samples) is a direct expression of changes in species composition across samples and spatial units within a given region, identifying actual β-diversity patterns from local to large scale is crucial to obtain reliable estimates of regional species richness (γ-diversity).

The total–species (T–S) accumulation curve proposed by Ugland et al. [4] and refined by Bevilacqua et al. [18] provided a robust methodological framework for estimating γ-diversity, taking into account small-scale patchiness, variations in β-diversity, and separate accumulation patterns for rare, intermediate, and common species within the region of interest. The modified approach of the T–S curve estimated 382 (95%CI: 358, 389) species of macroalgae on coralligenous outcrops in the whole investigated area, which represented a realistic upper limit of total species richness as it is higher than the observed richness across all samples ($S_{obs} = 187$) and did not exceed the maximum threshold of 487 species found in the region over two centuries of research. Species richness estimates from the T–S curve were also highly stable, irrespective of potential uncertainty in the measured total surface of outcrops or varying thresholds for species rarity. The refined T–S curve outperformed the other estimators, which returned inconsistent values of total species richness. Non-asymptotic parametric estimators largely overestimated the regional diversity of macroalgae on outcrops, providing estimates exceeding ~50% the maximum value in the best case (i.e., logarithmic accumulation model). In contrast, nonparametric and parametric asymptotic methods provided estimates very close to the observed total number of species in the samples. Although plausible, these estimates cast doubts on possible underestimations, as they returned only 3–38% more estimated species over the total area of the outcrops in the region, which was ~4.3 × 10^4 times larger than the sampled surface. Moreover, nonparametric estimators are generally tailored to estimate the number of species that may have been in a set of samples [7], rather than to infer the number of species over a large area, therefore producing conservative, lower-bound estimates [46,47].

Our investigation of macroalgal species richness on coralligenous outcrops confirmed that the accuracy of the T–S curve is not intrinsic but critically influenced by the spatial scale of analysis, and specifically by the subunits used in the model of species accumulation. In systems where sampling is robust and the target assemblage is well-known, the T–S curve delivers highly accurate estimates, often outperforming traditional nonparametric estimators, as observed for arbuscular mycorrhizal fungi [48] and native trees [49] in temperate forests, and for intertidal macrobenthic invertebrates [3]. However, when the chosen spatial subunits fail to capture the true β-diversity in the area, potential overestimation may occur [18]. This issue was observed in the study by O'Dea et al. [2] on birds, where T–S estimates were consistently high, and, similarly, in the work by Melo et al. [50], which showed a consistent overestimation of macrophyte richness. If the identified subareas to construct the T–S curve are still heterogeneous spatial units that can be divided to fit the actual β-diversity patterns, the model will tend to overestimate the regional species richness [18]. In this case, the residual spatial heterogeneity within subareas could inflate the rate of species accumulation in the T–S curve, leading to overestimated species richness. In our study, the finer partitioning into 6 subareas allowed reducing the overestimation with respect to the T–S curve based on the original 4 subareas by 11%. This subdivision of the total area is more aligned with β-diversity variations and therefore improved the extrapolation of regional γ-diversity.

Overestimation may also arise due to small-scale patchiness, which could affect the rates of species accumulation of the T–S curves and the resulting estimates [5,51]. In the case of nonrandom distribution of individuals or species at local scale, sampling units of limited surface to adequately sample the assemblage or organisms of interest could erroneously increase small-scale patchiness [13]. In these situations, a mis-assignment of α-diversity to β-diversity could occur [32], causing an overestimation of total species richness. In our case, the analysis did not highlight signs of inadequate sample size, such as extreme dissimilarity values among samples (i.e., 0 or 100% dissimilarity) or skewed frequency distribution of dissimilarity values in the dataset, and sample pooling was unnecessary. This is probably because our samples (0.75 m^2) were actually the result of pooled sampling units of smaller surface (i.e., 3 sampling units of 0.5 m^2), and large enough to effectively capture the local-scale diversity of macroalgae on coralligenous outcrops.

The largest contribution to enhance the performance of the T–S curve derived from the integration of species rarity in the accumulation model. The importance of weighting species rarity was already highlighted by Reichert et al. [3], suggesting that the performance of the T–S curve may be improved when the proportion of rare species is neither high nor low. Indeed, the extrapolation of the number of species from the T–S curve (and also other parametric estimators) assumes a continuous increase in species richness at increasing number of samples, which may not be necessarily true for all species. If the continuous increase can be hypothesized for rare species (<5% of samples), the accumulation of common (>10% of samples) and intermediate (5–10% of samples) species is very likely to saturate after accounting for a limited number of samples [18]. Therefore, considering rare, common and intermediate species altogether in the accumulation model would tend to largely overestimate γ-diversity, as the accumulation rate is extended to all species, irrespective of potential saturation of more frequent species. It worth noting also that different taxa may exhibit different accumulation rates [51], potentially causing the slope of T–S curve to be inflated and leading to an overestimation of total species richness, thus requiring a careful consideration of variations in species distribution among different taxonomic groups to avoid biased estimates.

5. Conclusions

Quantifying species richness at a regional scale is challenging, time-consuming, and costly [52]. However, the need to increase the knowledge on the overall species diversity within a given region has important practical implications concerning conservation strategies and impact assessments across large and environmentally complex marine regions [20], given the ongoing increase in anthropogenic pressures and the rapid climate-driven changes to marine biodiversity [53]. If environmental impact assessments targeting the effects of human activities on α-diversity are useful for local-scale management, evaluations of γ-diversity could help to understand the wider-scale consequences of biodiversity changes and to provide baseline information for conservation planning over large areas. From this perspective, implementing suitable tools to extrapolate the total number of species within a region from a limited set of samples is essential. We demonstrated that the T–S curve, refined to reduce the potential bias arising from erroneous quantification of small-scale patchiness and spatial heterogeneity in species composition in the region of interest, and from the overestimation of rare species, allows for better estimates of γ-diversity with respect to other species richness estimators. Such findings, which benefited from a study context where the actual number of species was virtually known, strongly support the validity of the T–S curve approach in providing reasonable extrapolation of regional species richness.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/environments12120490/s1, Figure S1: Species accumulation curve (n = 37, 4 subareas); Figure S2: Frequency distributions for Bray–Curtis pair-wise dissimilarities for the dataset at sample aggregation; Figure S3: Proportion of undefined and 100%-dissimilarity values in the multivariate data for increasing numbers of (randomized) original replicate samples being pooled; Figure S4: Species accumulation curve (n = 37, 6 subareas); Table S1. Species richness estimation bootstrap results for 10,000 replicates; Table S2: Summary of result of regression analysis for parametric estimators. Supplementary Material S1: Dataset.

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