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Content

Non-technical summary

Defining '*reference conditions*' (*sensu* the Water Framework Directive, hereafter WFD) in Mediterranean lagoons is challenging for three main reasons. Firstly, Mediterranean societies have used lagoons for hundreds of years: written and archaeological records dating back to Roman times document their uses – as naval ports (Averno, Amvrakikos Gulf), clam farming sites (Lucrino) etc. – and the human impact on them, including hydraulic modifications (Sabaudia) and chemical pollution from mining (Mar Menor). Therefore, pristine conditions are unlikely to exist in Mediterranean lagoons. Secondly, lagoons are naturally rich in nutrients and organic matter and are physically stressed, with strong and unstable internal gradients. A high degree of heterogeneity exists, both between and water bodies and within them. All the main abiotic sources of natural variability and related uncertainty thus have to be taken into account in Mediterranean lagoon status assessments in order to evaluate benchmark conditions. Thirdly, lagoons are small when compared to their input ecosystems, i.e., the marine and freshwater realms from which almost all lagoon species come. Therefore, species composition is naturally highly variable between lagoons, mostly due to simple lottery competition: i.e. with lots of potential colonisers but very few available slots, those who come first get the best places. Accounting for these peculiarities of Mediterranean lagoons, this study addressed two main issues in classifying its ecological status: testing the adequacy of the proposed typologies and defining type-specific reference conditions and related boundaries. Thus, using two WISER datasets we compared different approaches in order to identify the main sources of metric-specific uncertainty in Mediterranean lagoons, quantified their relevance, derived type-specific reference conditions and refined the boundaries for various assessment tools. The first of these datasets is based on a study of fourteen 'reference' lagoons (or lagoon areas) in the Mediterranean and Black Sea basins, considering different habitat types within each lagoon. The latter refers to a seasonal study of disturbed and undisturbed stations in Lesina lagoon carried out over two consecutive years. It is used to evaluate site-specific variability and propose methods to cope with it. Four multimetric indices used for benthic assessment were selected: BAT (Benthic Assessment Tool), BITS (Benthic Index based on Taxonomic Sufficiency), ISS (Index of Size Spectra) and M-AMBI (multivariate AMBI). The multimetric indices differ in terms of their general approach, being either taxonomic (BAT, BITS, M-AMBI) or non-taxonomic (ISS), and, in the case of the former, in terms of the level of taxonomic resolution, i.e. species (BAT and M-AMBI) or family (BITS).

Title: Methodological approaches to the definition of type-specific reference conditions for benthic macroinvertebrates in transitional waters: the case study of Mediterranean lagoons.

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Abstract

Ecological status is a measure of ecosystem health and is expressed as the distance from an '*optimal*' status, which is achieved when pressures from anthropic activities consuming energy subsides are completely absent. In the European Water Framework Directive (WFD), optimal status is referred to as '*reference conditions*', which encompass a degree of natural variability in terms of both structural and functional ecosystem components. Natural variability is particularly relevant to Mediterranean lagoons, which are characterised by strong and unstable internal gradients and are naturally rich in nutrients and organic matter. Lagoon typology (*sensu* WFD), habitat patchiness and seasonality are the main sources of natural variability and related uncertainty in lagoon status assessments. All assessment tools used to classify benthic ecological status, involving both single metrics and multimetric indices, are sensitive to some of the intrinsic sources of lagoon variability.

In this study we have considered a set of Mediterranean reference lagoons and the lagoon typologies so far proposed in order to: (i) explore the influence of spatial and temporal sources of natural variability on various multimetric assessment tools, i.e. BAT (Benthic Assessment Tool), BITS (Benthic Index based on Taxonomic Sufficiency), ISS (Index of Size Spectra) and M-AMBI (multivariate AZTI's Marine Biotic Index).; (ii) evaluate type and metric specific reference conditions and related classification boundaries; (iii) test the accuracy of both type-aspecific and type specific classification boundaries to evaluate ecological status of reference lagoons; and, (iv) propose recommendations to optimising ecological status classification in Mediterranean lagoon ecosystems. A validation test on an independent sample of Mediterranean lagoon sites is part of the study.

All assessment tools showed a large natural variation on the spatial scale among and within the reference lagoons (i.e., 30% ≤Coefficient of Variation≤ 65.8%) as well as on the temporal scale (i.e., 18% ≤CV≤ 39.1%). Surface area, tidal range, confinement and water salinity, which are the drivers of the Mediterranean lagoon typologies proposed so far, were actually found significant sources of the spatial variability of at least some of the considered assessment tools, with only water salinity significantly affecting all of them. Accordingly, a mixed model analysis was used to quantify a new metric-specific typology. Typologies accounting for the sources of natural variation significantly reduced the assessment tool variability; consequentely, the definition of type specific reference conditions and classification boundaries improved the accuracy of the ecological status assessment. However, at the study site level the new type specific reference conditions boundaries did not manage to prevent a certain degree of uncertainty or miss-evaluation in the assessment (proportion of sites assessed as moderate (M) to bad (B) at reference condition lagoons: 14.9. ≤%M-B sites≤ 37.6). At the lagoon level, the accuracy raised at 100% for the more complex typological schemes and remained as high as the 83.3 in a validation test performed on an independent set of strongly disturbed sites (expected ecological status from moderate to bad). Recommendations on the application of the different

proposed approaches to type and metric specific reference conditions and classification boundaries are given.

Key words

Reference conditions, Mediterranean lagoons, typology, habitat patchiness, seasonal variability, multimetric indices, benthic fauna, Water Framework Directive

1 Introduction

The Water Framework Directive (WFD, 2000/60/EC) requires EU Member States to assess the ecological status of each water body in Europe. Ecological status is an ecosystem property describing the overall organization, functioning and stability of ecosystems in terms of structural composition and dynamics as well as process rates and efficiencies. The quantification of ecological status presents some intrinsic conceptual difficulties, although some remarkable approaches have been proposed (Costanza, 1992; Jorgensen et al., 2005; Marques et al, 2009).

The WFD addresses this key issue at a comparative level, evaluating the actual ecological status of real aquatic ecosystems according to their distance from totally or near-totally undisturbed conditions, i.e. with little or no disturbance from human activities. This comparison is called Ecological Quality Ratio (EQR), in the WFD's terminology. Hence, the ecosystems enjoying pristine conditions can provide 'reference conditions' for such assessment (WFD, 2000; Borja et al., 2004a; Muxika et al., 2007). Similar approaches have been adopted by marine legislation worldwide and by the Marine Strategy Framework Directive (MSFD) in Europe (Borja et al., 2008, 2011). Accordingly, by definition the structural and functional characteristics of ecological communities and guilds, in addition to Biological Quality Elements (BQEs in accordance with WFD terminology), under reference conditions provide a quantitative description of high ecological status.

The definition of 'reference conditions' is particularly challenging for lagoon ecosystems, which are common and highly valuable (Costanza et al., 1977) habitat patches in the Mediterranean and Black Sea coastal landscape, covering an overall area of approx. 10000 km^2 (Transitional Water Platform, www.circlemednet.unisalento.it). In fact, lagoons, defined as "coastal water bodies receiving some freshwater inputs and divided from the sea by sand bars with one or more openings, which can be permanent or temporary" (Kjerfve, 1994), are naturally eutrophic ecotone ecosystems (Basset et al., 2011, in press) with high spatial and temporal variability.

According to the WFD the main options for establishing reference conditions are: (i) comparison with an existing undisturbed site or a site with minor disturbance; (ii) historical data and information; (iii) models; or (iv) expert judgment (Bald et al., 2005; Borja et al., 2004a). Following Borja et al. (2011), the advantages and disadvantages of each option, when applied to Mediterranean lagoons as a model for transitional waters, are shown in Table 1.

Table 1. Advantages and disadvantages of the four main methods for deriving reference conditions, recommended by the Water Framework Directive.

Methods	Advantages	Disadvantages
"Pristine" or undisturbed conditions	• High robustness • High accuracy • General applicability • Low data requirement • Simple to apply	• Undisturbed ecosystem areas are uncommon for most categories of water bodies • Unclear definition of the minimum level of acceptable disturbance
Historical data	• High transparency and comprehensibility	• Scarcity of pre-industrial data • Variability due to changing conditions not considered; • Low applicability
Models	• High robustness and reliability regarding functional aspects; • General applicability	• Large datasets are required for standardisation; • Low/medium robustness and reliability regarding taxonomical aspects; • Low transparency and comprehensibility;
Expert judgment	• High robustness and reliability • General applicability • Very practical and pragmatic	• Large datasets regarding pressures are required; • Early signs of disturbance are lost; • Low transparency and comprehensibility

In general, the description of reference conditions based on the characteristics of ecosystems undisturbed by anthropic activities is a difficult task, since pristine systems are scarce worldwide (Mitsch and Gosselink, 2000; Moss, 2007; Halpern et al., 2008). Large rivers, lowland rivers and almost all rivers in the Mediterranean basin have been so severely altered by hydraulic engineering, water abstraction, contamination and other impacts that undisturbed, pristine conditions do not exist any longer (Birk and Hering, 2009). The same difficulties in identifying pristine reference conditions are found for coastal and transitional waters (Nielsen et al., 2003; Jonge et al., 2006; Borja et al., 2004b). Transitional waters are the end points of freshwater networks and more than 70% of the total human population lives in coastal regions (Dauvin, 2007; Elliott and Quintino, 2007). Recorded impacts on Mediterranean lagoons, resulting from human activities, date back at least to the Roman Age (Bellotti et al., 2007; Bony et al., 2011; Elbaz-Poulichet et al., 2011) and the management of lagoon hydraulics was a common and widespread practice in Mediterranean lagoons to optimize their use for fishery activities (see De Giorgi, 1895, for the case of Lake Alimini in Southern Italy); therefore, it is very unlikely that completely pristine lagoons occur in the Mediterranean basin. On the other hand, in the last forty years, the disturbance impacts have locally been reduced and/or better controlled in many Mediterranean lagoons since their ecological relevance has been increasingly recognised by various international initiatives (e.g., Ramsar Convention, MedWet initiative, Habitat and Nature 2000 Directives). 'Coastal Lagoons' have been defined as priority habitats by the Natura 2000 Directive ("habitat 1150", as specified in the subsequent Directive 2006/105/EC) and many Mediterranean lagoons or lagoon areas have been included within protected areas. Therefore many lagoons might be considered with currently minor disturbance pressure, even though the use of definitions of minor disturbance pressures to set reference conditions for lagoons at the Mediterranean Ecoregional scale needs to account for a risk of bias due to the difficulty to achieve accurate enough comparisons of lagoon vulnerability and sensitivity at such a large spatial scale (Brik and Bohmer, 2007; Barbone et al., 2011).

When pristine conditions are not available, it is possible to use historical data (Nielsen et al., 2003). However, this approach also has some constraints. First of all, for most European countries pre-industrial historical data is not available (Borja et al., 2004b). Moreover, changes in the abiotic environment, setting the environmental niche (*sensu* Zobel, 1997) for biological life, can be irreversible. Climate change is the most immediate example, but the dispersion of species into un-saturated communities is also a common event in aquatic ecosystems (Table 1). Finally, historical conditions may not be socially acceptable as reference conditions, since the improvement of our quality of life has entailed a level of resource exploitation, which cannot be reduced in the short term without causing major socio-economic problems. The exploitation of water resources, including ground water, for domestic and industrial uses is the most obvious example. Moreover, as regards Mediterranean lagoons strong disturbance pressures can be dated back to much earlier times than those of the first quantitative biological and ecological assessments of their flora and fauna.

Modelling reference conditions using auto-ecological data has also been used (Nielsen et al., 2003; Meyer et al., 2008). Modelling has been developed particularly in streams and rivers, following the RIVPACS and RIVPACS-type approaches (Wright et al., 1994; Moss et al., 1987; Wright, 2000; Clarke et al., 2003; Kokes et al., 2006). However, actual reference conditions are required to calibrate the model, leaving the field open to criticism of a lack of reference conditions for most aquatic ecosystem types and categories in Europe (Table 1). Accordingly, this approach was not yet applied to Mediterranean lagoons and clear constraints to its application have been already emphasised here.

The use of 'virtual' reference locations, such as those ecosystems least impacted by anthropogenic pressures, and of an 'expert judgement' approach has been proposed by Borja et al. (2004a) and Bald et al. (2005). They have been used successfully in macroinvertebrate status assessment (Rosenberg et al., 2004; Muxika et al., 2007; Borja et al., 2009a; Barbone et al., 2011). An integration of the 'Pristine ecosystem' and 'Expert judgment' approaches can be used to define reference conditions with reference to least disturbed conditions (Birk and van Kouven, 2009), setting a common and objectively assessable boundary between "high" and "good" status (CIS Guidance document 14, EC, 2011).

Finally, objective methods to derive reference conditions '*a posteriori'* from extensive datasets covering regional areas have also been developed recently (Borja et al., 2009b; Pinto et al., 2009; Borja and Tunberg, 2011), assessing the adequacy as reference conditions of sites with the highest observed ecological status on the basis of specific metrics. In accordance with this approach, type-specific reference conditions have also been published, but only for M-AMBI (multivariate AZTI's Marine Biotic Index) (Muxika et al., 2007; Borja et al., 2009b; Borja and Tunberg, 2011, Barbone et al., 2011), ISS (Index of Size spectra Sensitivity) (Barbone et al., 2011) and to some extent for the British, Irish, Danish and Norwegian methods (Borja et al., 2007).

The definition of type specific reference condition is a main building block of the WFD construction. The need to establish type-specific biological reference conditions results from the dependency of the biological colonisation of aquatic ecosystems on the abiotic context (i.e., the niche width of the environment, Emlen, 1973), which filtres a set of actual colonisers among the much larger pool of potential ones (Zobel, 1997). Type specific reference conditions must summarize the range of possibilities and values for biological quality elements under totally or near-totally undisturbed conditions, over periods of time and across the geographical range of potentially significant types (Vincent et al., 2002). The identification of type-specific reference conditions is a good solution to the non-linearity of ecosystem functioning components with ecosystem ecological status.

1.1. The spatial component of reference condition heterogeneity: lagoon types and habitat types within lagoons

The heterogeneity of environmental conditions in Mediterranean lagoons, which requires the clustering of lagoons into lagoon types, has two main components: (i) an inter-lagoon component, which is accounted for by a classification of lagoons into lagoon types (*sensu* WFD, 2000) depending on the most significant sources of variation, and (ii) an intra-lagoon component, which arises from habitat patchiness and seasonality and represents a major source of uncertainty in the assessment of type-specific reference conditions.

In order to minimize intra-type variation, which can lead to an inaccurate classification of ecological status, lagoon typology needs to identify the major sources of variation among the descriptors (mainly abundance, richness and diversity) of biological quality elements (i.e. phytoplankton, macrophytes, benthic invertebrates, fish; WFD, 2000).

The first attempt to develop a general framework to describe the spatial heterogeneity of Mediterranean lagoons dates back to the Venice System (Battaglia, 1959) and confinement theory (Guelorget and Perthuisot, 1983). However, only the former dealt with inter-lagoon heterogeneity, highlighting the role of water salinity and organizing lagoons into 5 salinity classes, from oligohaline (water salinity lower than 5 psu) to hyperhaline (water salinity higher than 40 psu). Confinement theory was the first systematic attempt to deal with habitat patchiness inside lagoons, classifying habitats into zones according to water residence times, from fully mixed (close to the lagoon inlet with strong marine influence) to confined (far from the lagoon inlet, either oligohaline or hyperhaline). The degree of confinement was also considered among lagoons, leading to a classification of these ecosystems in leaky, restricted and chocked (Kjerfve, 1994) along a gradient of decreasing hydraulic openness.

More recent and specific approaches to identify the most relevant sources of variation of benthic macroinvertebrates in Mediterranean lagoons have highlighted the importance of lagoon surface area (Basset et al., 2006; Basset et al., 2007), tidal range (Basset et al., 2006; Basset et al., 2007; Barbone and Basset, 2010), water salinity (Boix et al., 2005; Basset et al., 2007; Lucena-Moya et al., 2009; Barbone et al., 2012) and the presence of aquatic flora (Barbone et al., 2011).

The relevance of lagoon surface area as a major factor in lagoon typology has also been observed for atoll lagoons (Dufour and Harmelin-Vivien, 1997; Andrefouet et al., 2001), following island biogeography theory and Species-Area Relationships (SARs), which have been observed for benthic macroinvertebrate species at various levels of taxonomic resolution (Sabetta et al., 2007). Quantitative water exchange between lagoons and coastal marine ecosystems has been found to be particularly relevant to Italian lagoons (Barbone and Basset, 2010), regarding differences between them in the degree of openness (Barbone et al., 2012) and tidal hydraulic forces, with tidal range ranging from few centimetres to more than 1 m. With regard to salinity, a varying number of salinity classes have been proposed so far for Mediterranean lagoons. These range from the five classes of the Venice System (Battaglia, 1959; Basset et al., 2007) to the three classes proposed for the Balearic Island lagoons in Spain (Lucena-Moya et al., 2009). The latter were classified into oligohaline (up to 5 ppt of salinity), mesohaline (6–26) and euhaline (>26).

Surface area, degree of confinement (related to openness), tidal range, and salinity have been quantified as thresholds by the Mediterranean countries (Table 2). However, these are assigning different weightings by different countries in the definition of their national transitional water typologies. Surface area, tidal range and salinity are considered in the national lagoon typology of Italy (ISPRA, 2007), where salinity thresholds are derived from the Venice System (Battaglia, 1959) and tidal range and lagoon surface area are set with reference to the distribution of values in the full set of Italian lagoons (Basset et al., 2006). Overall, 20 lagoon types have been selected by Italy (based on 2 levels of tidal range, 2 of surface area and 5 of water salinity; ISPRA, 2007), which are grouped into 3 major macro-types (ISPRA 2010). On the other hand, in its national transitional water typology Greece has considered a single lagoon type, i.e. "Coastal Lagoons" (HCMR, 2010; *internal document*). Italy has also recently re-organized its national typology in its water monitoring legislation (DM 56/2008, DM 131/2008, DM 260/2010) considering a single lagoon type, i.e. "Coastal Lagoons" for non-tidal lagoons (*sensu* Basset et al., 2006). In its definition of Mediterranean lagoon typology, the MEDGIG project regarding benthic macroinvertebrates also includes the concept of confinement, classifying lagoons into leaky, restricted and choked, combined with water salinity in a two-factor classification system (Reizopoulou *pers. communication*).

The main advantages and disadvantages of the various factors considered in Mediterranean lagoon typology are shown in Table 2.

Table 2. List of advantages and disadvantages of main drivers used to define Mediterranean transitional water typology.

Driver	Advantages	Disadvantages
Confinement	• Solid theoretical foundations on qualitative variations at the intra-ecosystem level; • Well-defined expected patterns of structural and functional changes within lagoons	• Low transparency and comprehensibility in setting quantitative thresholds between types; • Stronger variability within than between lagoons; • Potentially confusing effect of co-variation with salinity; • Poor availability of data
Salinity	• Solid theoretical foudations and well-defined influence on species' functional traits; • Well-defined thresholds between types; • Large availability of data	• Uncertain relationships with simple metrics required by the WFD
Surface area	• Solid theoretical foundations; • Large availability of data; • Well-defined relationships with species richness	• Low transparency and comprehensibility in setting thresholds among types; • Uncertain relationships with WFD-required metrics other than species richness
Tidal Range	• Strong influence on lagoon ecosystem properties; • Strong influence on lagoon environmental niche	• Low transparency and comprehensibility in setting thresholds between types in the Mediterranean; • Uncertain relationships with WFD-required metrics other than species richness • Poor availability of comparative data on WFD-required metrics

Even with a detailed typology with many different types, significant spatial heterogeneity in terms of physico-chemical and hydro-morphologic characteristics would still occur within each lagoon, typically resulting in a mosaic of different habitats (Escavarage et al., 2004). Factors determining submerged landscape patchiness in Mediterranean lagoons, such as sediment composition, organic matter content, depth and vegetation cover, are known to affect species distribution (Ysebaert et al., 2003; Blanchet et al., 2004). Sediment grain size and organic carbon (Teske and Wooldridge, 2003; Reizopoulou and Nicolaidou, 2004), the presence and abundance of benthic macrophytes (Kafanov and Plekhov, 2001; Galuppo et al.2007; Arocena, 2007), macrophyte type (seagrasses vs. seaweeds; Galuppo et al. 2007) and water depth (De Casabianca and Posada, 1998) are known to affect macroinvertebtrate distribution, abundance and body size (Basset et al., 2007; Basset et al., 2008). Macrophytes have a major influence on the structure of submerged lagoon landscapes, affecting benthic life, since they interfere with the hydrodynamic regime, stabilize sediments, oxygenate the water, favour organic matter deposition and accumulation and provide a substratum, refuge and food for benthic invertebrates.

Natural variability of physico-chemical conditions also occurs within lagoons. Strong salinity gradients, variable with the season (see section 1.2), occur within lagoons at the freshwater-lagoon-marine ecosystem boundaries. Guelorget and Perthuisot, (1983) and Bianchi (1992) quantified the influence of these salinity gradients on the internal spatial patchiness of lagoons having the six zones (or more if freshwater and hyperhaline confined zones are taken into account; Bianchi, 1992) of confinement theory. Partially related to salinity gradients, oxygen content gradients, related to the organic content of sediment and fast decomposition rates particularly in hot periods (Sangiorgio et al., 2008), also have a strong influence on macroinvertebrate richness, abundance and body size (Basset et al., 2008).

At an indirect level, hydraulic forces, which shape lagoon physiography and the lagoon bottom landscape, have been observed to explain a significant component of macroinvertebrate heterogeneity in a sample of Mediterranean lagoons (Barbone and Basset, 2010). An analysis of the main abiotic drivers of a selection of multimetric indices using a mixed model approach has recently been developed (Barbone et al., 2011).

1.2. The temporal component of reference condition heterogeneity: the seasonality of structural lagoon components

The Mediterranean climate, with wet cold winters and dry or very dry hot summers determines high seasonal heterogeneity of physical and chemical conditions in Mediterranean lagoons. These conditions represent the environmental niche space for potential colonizers, driving the turnover of species and keystone functional groups such as juvenile fish (Maci and Basset, 2009) and migratory birds (Robledano et al., 2010). The Mediterranean climate also induces seasonality in rates of both primary production and organic matter decomposition, which directly affects benthic macroinvertebrates. Indeed, whilst most of the organic matter enters the system during the winter period, low winter temperatures reduce the rate of microorganism growth and decomposition efficiency (Sangiorgio, et al., 2008). This delays most of the detrital energy dissipation until the warmer spring period. As already shown for freshwater invertebrates in temperate rivers (Cummins et al., 1989), benthic invertebrate life cycles are likely to be optimized to fit the actual flow of available energy from decomposition processes. Therefore, changes in limiting environmental niche dimensions produce large shifts of niche position, reflected in their effect on the temporal distribution of species, functional groups and size classes in Mediterranean lagoons.

Seasonal fluctuations in the abundance and composition of benthic macroinvertebrate guilds arising from the occurrence of extreme environmental conditions such as low or high temperatures, floods and droughts have frequently been observed (Alden et al., 1997; Attrill & Power, 2000; Salen-Picard & Arlhac, 2002). Cold winters have direct and indirect effects on benthic macroinvertebrates, resulting in a decrease in species number, abundance and biomass (Reiss et al., 2006). Direct negative influences mainly include enhanced individual mortality, while indirect negative influences may include reduced reproduction and production (Reiss et al. 2006).

Salinity variation occurring at different scales within lagoon ecosystems (Comin et al., 2004) determines patterns of spatial variation in species distribution within the lagoon ecosystem as well as enhanced species turnover. Similar and even stronger alteration results from extreme events of precipitation and flooding (Boesch, 1977; Chainho et al., 2006), which cause strong seasonal changes in freshwater discharge and may cause huge variations in salinity (Comin et al., 2004). Consequently, this can profoundly alter the structure of a community.

Other natural disturbances occurring occasionally during the summer season include oxygen depletion and hypoxia or anoxia (Reiss and Kroncke, 2005; Chainho et al., 2007). Deviations in dissolved oxygen dynamics from the general temporal pattern have been attributed to environmental variables such as temperature, irradiance, precipitation, tides, freshwater discharge and wind (Beck and Bruland 2000). Several authors have reported that extreme hypoxic events occur in association with early morning low tides (Edwards *et al*. 2004), heavy cloud cover (D'Avanzo and Kremer 1994; D'Avanzo *et al*. 1996), calm wind conditions, usually following a rain event (Gallegos *et al*. 1992; Lapointe and Matzie 1996) and wind-driven mixing (Vignes et al., 2009). During these events there is a reduction of macroinvertebrate richness, abundance and body size (Lardicci et al., 2001; Basset et al., submitted).

1.3. Approaches to the definition of type-specific reference conditions in Mediterranean lagoons

Two different approaches can be followed in order to define type-specific reference conditions in Mediterranean lagoons.

The first approach is simply related to lagoon typology, as approved at the national level by Member States. Type-specific reference conditions are derived using a sub-set of lagoons for each type, which are classifiable as reference ecosystems in accordance with the procedures described in section 1.1, in order to assess the reference conditions of the selected assessment tools (Table 3). This approach, which is fully compliant with the WFD, considers typology as metric-aspecific, implicitly assuming that all multimetric indices used as assessment tools weigh potential sources of natural variability in Mediterranean lagoons in exactly the same way.

Table 3. Approaches to the definition of Mediterranean lagoon types

National Typologies *(a priori approach)*		**Optimised Typology** *(a posteriori approach)*
A - Type a-Specific Metric a-Specific	**B - Type Specific Metric a-Specific**	**C - Type Specific Metric Specific**
One Type "Lagoons"	Many Types factorially combining drivers	Many Types resulting from mixed model analysis of drivers
	B1 - Confinement + Salinity	Potentially different Types for different metrics
	B2 - Salinity + Surface area + Tidal range	

An alternative approach, which is also compliant with the WFD, but implies a more complex definition of typology, is developed by deriving the optimal classification of lagoon typology from an analysis of those environmental factors (i.e., environmental lagoon niche dimensions) that significantly reduce the uncertainty of the assessment in reference condition ecosystems. In this latter case, type-specific reference conditions might also be metric-specific, since uncertainty in the assessment of ecological status by means of multimetric indices can in principle be minimised by accounting for a number of environmental factors. In this alternative approach, environmental factors included in the proposals for a Mediterranean lagoon typology, as well as those determining most of the spatial and temporal landscape heterogeneity within lagoon ecosystems, should be taken into consideration as potential sources of macroinvertebrate assessment metric variation. Mixed-model procedures have already been used to quantify the most significant sources of assessment metric variation in reference ecosystems/sites and to derive type-specific reference conditions for them (Barbone *et al*., 2012). This approach can also be considered as an *a posteriori* validation of the national typologies, which are generally defined in accordance with an 'expert judgement' (Table 3).

Following these considerations, the present investigation has the following objectives:

(1) to evaluate of the accuracy of the current approaches to Mediterranean lagoon typology in the definition of type-specific reference conditions for both taxonomically-based and non-taxonomically based multimetric indices;

(2) to highlight the most relevant environmental sources of assessment tool variability in Mediterranean lagoons;

(3) to propose a methodological approach to set type-specific classification boundaries for a sample of the most frequently used assessment tools;

(4) to test the accuracy of the new type-specific boundaries on the classification of Mediterranean lagoon reference sites using an independent validation set of lagoons/lagoon areas;

(5) to evaluate the degree of variability of the classification tools at the study site scale in two-year sampling series.

Here, these objectives were addressed using two data sets made available to WISER for this purpose, one focusing on objectives 1 to 4 and the second focusing on testing objective 5.

2 Materials and methods

2.1. Study sites

2.1.1. Mediterranean and Black Sea lagoons

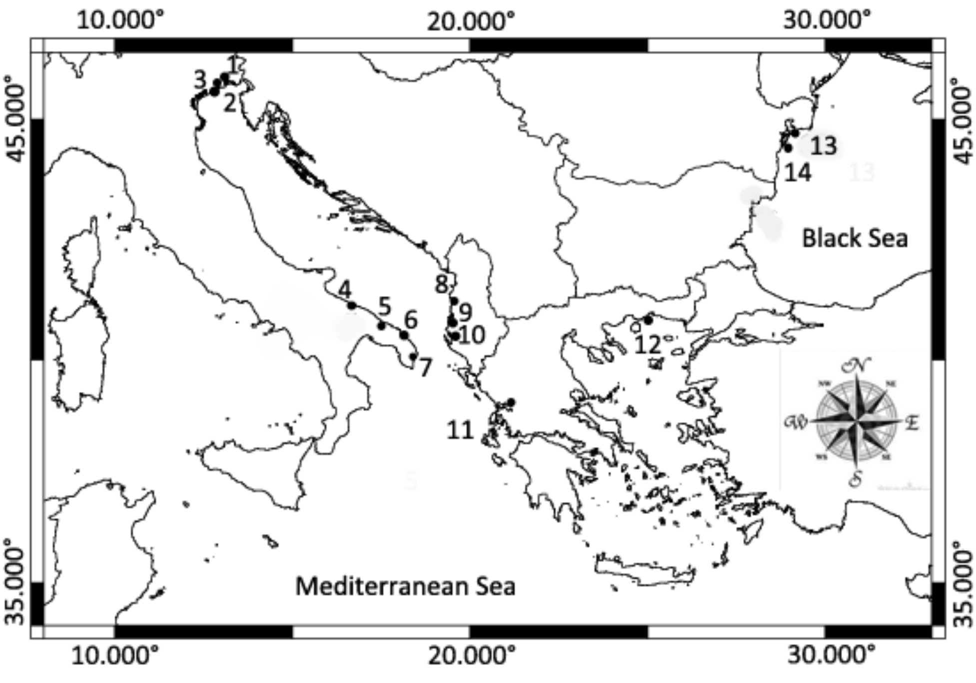
Data on benthic macroinvertebrates were originally collected from 14 lagoons located in 4 different countries: (from west to east) Italy, Albania, Greece and Romania. Data were collected during the TWReferenceNET Project (Basset et al., 2008), as part of the INTERREG IIIB CADSES (Central European, Adriatic, Danubian, South-Eastern, European Space) Program (Figure 1).

The group of studied ecosystems was selected according to two main criteria: (i) lagoon ecosystems or restricted areas inside lagoons with low human disturbance, considered as reference conditions for Eastern Mediterranean and Black Sea lagoons (see Section 1.3);

and, (ii) a wide range of heterogeneity for key abiotic variables in Eastern Mediterranean and Black Sea lagoons.

TWReferenceNet data are adequate to achieve the aim of this investigation, since the project was designed to compare macroinvertebrate metrics in reference and disturbed water bodies and along a stress gradient in each disturbed water body. Therefore the dataset includes a number of lagoons that can be considered as 'reference lagoons' for the Mediterranean and Black Sea ecoregion, identified by integrating the 'Pristine ecosystem' and the 'Expert view judgment' options. Within most disturbed water bodies, the pressures occurring at the first level of the selected gradient were comparable to those occurring at field stations within water bodies considered as 'reference lagoon ecosystems'; therefore, field stations at the first gradient level of disturbed ecosystems were included as undisturbed reference sites. None of the TWReferenceNet water bodies are completely pristine, but they were selected among the least impacted in the EcoRegional area.

Figure 1. Map of study sites. 1: Grado Marano Lagoon, 2. Grado 'Valle Cavanata', 3. Grado fish farm, 4. Margherita di Savoia Salt pans, 5. Torre Guaceto brackish Wetland, 6. Le Cesine brackish Wetland;, 7. Lake Alimini, 8. Patok Lagoon, 9. Karavasta Lagoon, 10. Narta Lagoon 11. Logarou Lagoon, 12. Agiasma Lagoon, 13. Sinoe Lagoon, 14. Leahova Lagoon.



The criteria for the selection of reference ecosystems were the inclusion of the ecosystem, or of part of it, inside a protected area and the result of an expert assessment of the degree of pressures on the ecosystem, i.e. ecosystem vulnerability. We based the first screening on the lagoon's occurrence inside a protected area on the basis of two major considerations: (i) all

Mediterranean and Black Sea lagoons have been exploited and modified by human activities but many of them now have relatively low pressures; and (ii) the occurrence within a protected area is a guarantee that actual pressures are at least controlled and in most cases minimised and that a management plan has been developed, aimed at the conservation of good environmental status for the whole lagoon landscape. Within ecosystems otherwise affected by varying degrees of anthropogenic pressure, a few relatively pristine areas and/or areas that are highly valuable for conservation purposes (e.g., Ramsar areas, Sites of Community Interest, Special Protected Areas, Regional/National Parks) were considered for this study as 'reference sites'. In addition, a quantitative assessment of pressures acting on each ecosystem was performed during the TWReferenceNet project with expert quantification of the external pressures, supporting the criteria used for the 'reference ecosystems' selection procedure; low perturbation pressures for the ecoregional areas were ascertained using a 'expert view' approach (Table 4). Nevertheless, for the some of the selected study sites, there is evidence of localized disturbance events from specific sources (e.g., Piacenti et al, 2000; Ciubuc, 2004; Cullaj et al., 2005; Christophoridis 2007). The disturbed lagoons in the TWReferenceNet data base and as well the most disturbed areas within lagoons were also utilised as an independent set of lagoons/lagoon areas for validation purposes of the new type-specific boundaries derived from the 'reference lagoons and sites'. The expected classification for these disturbed lagoons in the validation test was within the moderate/poor/bad classes.

Considering the second criterion for study site selection, i.e. their representativeness of lagoon niche space, the selected lagoons varied from micro-tidal to non-tidal, large to small, hyperhaline to oligohaline and choked to restricted (Kjerfve and Magill, 1989; Basset et al., 2006) (Table 5). Moreover, they lay within two bio-geographical areas represented by the Eastern Mediterranean and Black Sea ecoregions. Therefore, even though some of the most important and well-studied Mediterranean lagoons, as Venice lagoon, Vaccares, or the Ebro delta lagoons, were not among the lagoons selected for this study, the latter cover a large range of the abiotic variability of Mediterranean lagoons. Within each ecosystem, additional sources of variability that were known to affect the performance and distribution of benthic fauna were taken into account. These included spatial components such as habitat patchiness as well as temporal components. In this case, in order to account for habitat patchiness, sites were selected from two or three dominant habitat types defined by a factorial classification of sediment granulometry and vegetation cover/type as in Basset et al. (2008). The temporal component was addressed by sampling both before and after summer, which may represent a critical period of discontinuity in Eastern Mediterranean and Black Sea lagoons.

Overall, 101 study sites, considered here as reference sites, were sampled within the 14 lagoons with 2/3 habitat types per reference lagoon, 2 sites per habitat type and 5 replicates per site; at the first level of pressure of disturbed lagoons only one site per lagoon, with 5 replicates, was sampled. The same experimental design was applied at additional 57 study sites, considered here as disturbed sites.

Table 4. Pressure evaluation for transitional water ecosystems considered. Intensity of each pressure type was evaluated using scale from 0 to 4 (0=absent; 1=very low; 2=low; 3=moderate; 4=high) in accordance with expert evaluation based on existing knowledge as reported in TWReferenceNet report. A = organic load; B = nutrient load; C = hazard substances; D[a] = fishing; E = alien species; F = navigation; G= physical modification; H= average pressure; I[a] = net pressure.

Transitional waters	Pressures									References
	A	B	C	D*	E	F	G	H	I*	
Agiasma	2	2	1	3	-	-	2	2.0	1.7	Nicolaidou et al., 2006; Christophoridis et al., 2007; Orfanidis et al., 2010.
Logarou	1	2	1	4	-	-	3	2.2	1.7	Komas et al., 2001; Reizopoulou and Nicolaidou, 2004; Nicolaidou et al., 2006; Christia and Papastergiadou, 2006;
Alimini	1	1	-	3	1	-	1	1.4	1.0	Basset et al., 2008.
Grado Marano[b]	2	2	3	-	2	3	2	2.3	2.3	Ianni et al., 2008; Basset et al., 2008; Ponti et al., 2008; Facca and Sfriso, 2009.
Grado Valle Cavanata	1	1	1	-	1	-	1	1.0	1.0	Basset et al., 2008;
Grado Valli da Pesca[b]	1	1	-	4	4	-	2	2.4	2.0	Basset et al., 2008;
Le Cesine	-	1	-	-	-	-	2	1.5	1.5	Basset et al., 2008;
Margherita di Savoia[b]	2	2	-	1	-	-	4	2.2	2.6	Basset et al., 2008;
Torre Guaceto	-	1	1	-	-	-	1	1.0	1.0	Basset et al., 2008;
Karavasta	1	1	-	4	1	1	3	1.8	1.4	Cullaj et al., 2005; Xhulaj and Miho, 2008; Beqiraj at al., 2007;
Narta[b]	2	2	-	3	-	-	4	2.7	2.6	Cullaj et al., 2005; Xhulaj and Miho, 2008; Beqiraj at al., 2007;
Patoku	-	-	-	2	1	-	1	1.3	1.0	Cullaj et al., 2005; Xhulaj and Miho, 2008; Beqiraj at al., 2007;
Lehaova	1	1	-	1	1	-	1	1.0	1.0	Basset et al., 2008;
Sinoe	1	1	-	3		-	3	2.0	1.6	Basset et al., 2008;

[a] Fishing pressures are not considered so effective on benthic macroinvertebrates. Net Pressures exclude fishing.

[b] Disturbed ecosystems have internal disturbance gradients; less disturbed sites can be estimated to undergo 33% of Net pressures.

Considering the second criterion for study site selection, i.e. their representativeness of lagoon niche space, the selected lagoons varied from micro-tidal to non-tidal, large to small, hyperhaline to oligohaline and choked to restricted (Kjerfve and Magill, 1989; Basset et al., 2006) (Table 5). Moreover, they lay within two bio-geographical areas represented by the Eastern Mediterranean and Black Sea ecoregions. Therefore, even though some of the most important and well-studied Mediterranean lagoons, as Venice lagoon, Vaccares, or the Ebro delta lagoons, were not among the lagoons selected for this study, the latter cover a large range of the abiotic variability of Mediterranean lagoons. Within each ecosystem, additional sources of variability that were known to affect the performance and distribution of benthic fauna were taken into account. These included spatial components such as habitat patchiness as well as temporal components. In this case, in order to account for habitat patchiness, sites were selected from two or three dominant habitat types defined by a factorial classification of sediment granulometry and vegetation cover/type as in Basset et al. (2008). The temporal component was addressed by sampling both before and after summer, which may represent a critical period of discontinuity in Eastern Mediterranean and Black Sea lagoons.

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2.1.2. Lesina lagoon

It is located on the southern Adriatic coast of Italy and is characterised by shallow waters (0.7–1.5 m) and limited exchange with the sea, features that it shares with many other Mediterranean lagoons. The lagoon is about 24.4 km long, with a total area of 51.4 km^2; the catchment area is about 600 km^2. The lagoon is connected to the sea by two narrow artificial canals: Acquarotta to the West, about 2 km long, and Schiapparo to the East, about 1 km long (Figure 2). On the basis of the position of the outlets and the lagoon's physiography, Lesina may be divided into three water bodies: western, central and eastern (Figure 2).

Figure 2. Map of Lesina lagoon study site.

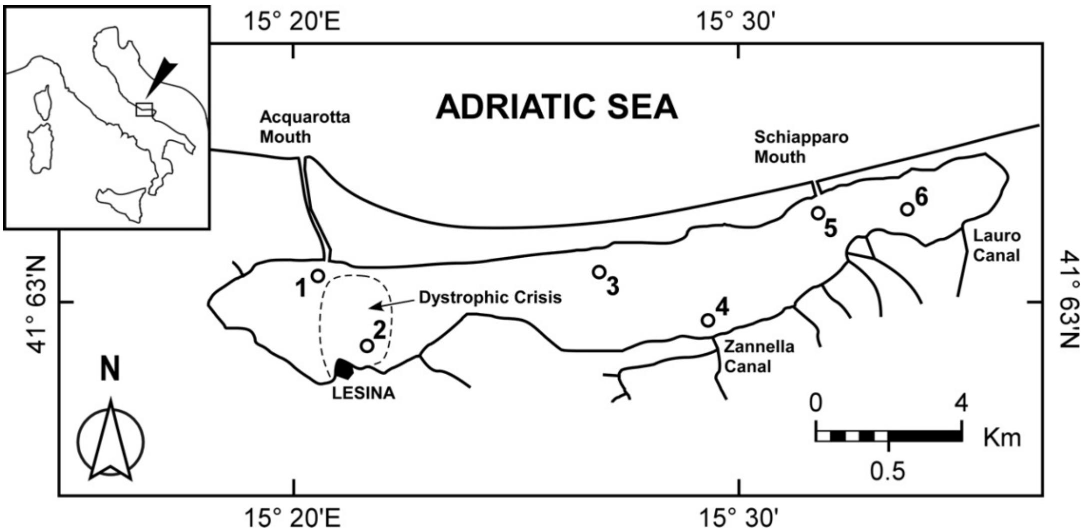


Table 5. List of studied transitional water eco systems showing geographical position and potential sources of inter and intra ecosystem heterogeneity.

TW's id	Transitional Waters	Country	Protection	Coordinates		Surface	Salinity	Confinement	Vegetation	Sediment	Depth (cm)		Oxygen (mg/l)	
				Lat.	Long.	(km^2)					Mean	Std.er.	Mean	Std.er.
1	Grado Marano	Italy	c	45.742°	13.221°	142.00	2; 3	2	2	1	117.5	28.4	8.3	0.05
2	Grado Valle Cavanata	Italy	a,b	45.712°	13.470°	2.10	3	2	1	1	90.0	5.8	7.6	0.1
3	Grado Valli da Pesca	Italy	c	45.722°	13.362°	0.30	3; 4	2	2; 3	1	61.3	8.1	8.3	0.5
4	Margherita di Savoia	Italy	a,b,c	41.429°	15.988°	12.00	4	1	2; 3	1	38.8	2.3	8.1	0.5
5	Torre Guaceto	Italy	a	40.711°	17.795°	1.60	2	1	1; 3	1	45.0	2.0	5.4	0.8
6	Cesine	Italy	a,b	40.358°	18.335°	0.70	1; 2	1	1; 3	1, 2	108.3	12.0	8.7	0.3
7	Alimini	Italy	b,c	40.202°	18.446°	1.40	3; 4	1	1; 2	1, 2	106.7	14.8	7.9	0.2
8	Patok	Albania	b,c	41.635°	19.590°	7.10	3; 4	2	2; 3	1	50.0	6.0	9.4	0.5
9	Karavasta	Albania	a	40.920°	19.472°	45.00	4	1	2; 3	1	120.0	10.0	9.6	0.6
10	Narta	Albania	c	40.529°	18.426°	29.90	4	1	2	1	47.5	7.5	9.7	1.5
11	Logarou	Greece	b	39.062°	20.900°	24.20	3; 4	1	2	1	115.0	26.0	6.9	1.0
12	Agiasma	Greece	c	40.880°	24.620°	3.20	3; 4	1	3	1	70.0	2.7	7.1	0.6
13	Sinoe	Romania	a	44.620°	28.888°	129.60	1	1	1; 2	1, 2	54.2	9.5	8.8	0.4
14	Leahova	Romania	a	44.727°	29.028°	22.90	1	1	1; 2	1	84.0	16.6	7.7	1.0

Salinity: 1 = oligohaline (<0.5 PSU); 2 = mesohaline (5-18 PSU); 3 = polyhaline (18-30 PSU); 4 = euhaline (>30 PSU). Confinement: 1 = choked; 2 = restricted; based on lagoon openness. Vegetation: 1 = emerged vegetation; 2 = absence of vegetation; 3 = submerged vegetation. Sediment: 1 = mud; 2 = sand. Protection is classified as: a = Ramsar site; b = Natura 2000 site; c = Local protection plan.

The lagoon's main hydrological features, temperature and salinity follow a seasonal trend, with minimum values in winter and maximum values in summer. The moderate water exchange with the sea and the combination of seawater and freshwater inflows produce a salinity gradient in the lagoon with decreasing values from west to east. Freshwater inflows are provided by seasonal streams, which are mostly located in the eastern area of the lagoon. The partially treated waste-waters of three municipalities with a total of 30,000 inhabitants are discharged into the lagoon by the Elce channel, the river Lauro and the Idrovora Lauro pumping station. Waters drained from nearby cultivated land (21000 ha) are discharged into the lagoon via the Lauro and Zannella watercourses and the Idrovora Pilla pumping station. The area surrounding the western end of the basin is used for intensive vegetable and wheat farming. In addition, there are three intensive fish farms along the coast, which discharge wastewaters into the lagoon through small channels on the western side and through the Zannella channel on the eastern side. Finally there is a cattle farm situated on the western shore, near the Acquarotta channel.

The selected sites were classified as disturbed and undisturbed on the basis of the *a posteriori* analysis and evaluation of anthropogenic pressures in the catchment area using an 'expert judgment' approach (Table 6). The pressure index, calculated as the average value of the pressures, has higher values at the stations in sites 1 and 2 than the others (Table 6). Based on these results we consider sites 1 and 2 as potentially disturbed and the others as internal reference points. The analysis performed here sought to detect the natural seasonal boundaries of assessment metric variability inside the lagoon in order to refine the type-specific reference conditions accounting for potential intrinsic seasonal variability.

Table 6. Nutrient values and pressure evaluation for sampling stations. Intensity of each pressure type was evaluated using scale of values from 1 to 4 (0=absent; 1=very low; 2=low; 3=moderate; 4=high in accordance with expert evaluation based on existing knowledge. A= diffuse agricultural inputs; B= domestic discharges; C= industrial discharges; D= fin-fisheries; E= pressure index, calculated as mean value of pressures.

			Pressures				
Station	DIN(μM)	P-tot(μM)	A	B	C	D	E
1	4.8	0.4	3	2	3	4	3.0
2	5.7	1.3	3	3	3	4	3.3
3	5.8	0.7	2	0	0	4	1.5
4	5.5	0.5	2	0	2	4	2.0
5	8.2	0.4	2	2	2	4	2.5
6	5.4	0.9	4	2	2	2	2.5

2.2. Sampling procedures and analysis of samples

2.2.1. Mediterranean and Black Sea lagoons

Sampling campaigns were performed in autumn 2004 and spring 2005 (temporal component) in all selected ecosystems. Sampling sites in each ecosystem were chosen so as to include a variety of habitat types (mud/sand, with/without submerged and emerged vegetation), levels of lagoon confinement (choked/ restricted) and depths (habitat patchiness) (Table 4).

For each sampling site five replicates were collected by a manual Reineck box-corer (0.03 m^2). Each sample was sieved through a 0.5 mm mesh and the remaining material was preserved *in toto* in jars containing 4% buffered formaldehyde in seawater. In the laboratory, benthic samples were sorted under a stereomicroscope. All individuals were then identified to the highest possible degree of taxonomic resolution, counted, measured individually (total length for most taxa) to the nearest 0.01 mm using an image analysis device (Leica Qwin) and weighed to the nearest 1 µg after drying for 72 h at 60°C. Ash content was determined by combustion in a muffle furnace for 24 h at 500°C. Large individuals were combusted singly, whereas smaller animals were combusted in groups of con-specific individuals, as far as possible belonging to the same size class. All individual body size data were then expressed as individual biomass after calculation of the individual Ash-Free Dry Mass (AFDM). On a small subset of individuals, less than 5% of the overall sample, for which it was not possible achieve a direct measure of dry weight and ash content, AFDM was derived from the length-mass relationship computed at the population level. In total 31,569 individuals were identified, and individual body size was assessed for 99.1% of the entire sample. Less than 1% of all sampled individuals were not considered for the ISS index because less than two thirds of the individual body was available for measurement (Basset et al., 2011).

Chemical-physical water parameters (water salinity, dissolved oxygen and temperature) were monitored close to the bottom at each station during sampling activities using a hand-held multi-probe (YSI 556).

2.2.2. Lesina lagoon

Data refer to six sites, two located in the western basin, three in the central and one in the east (Figure 2). The sampling campaigns were carried out on a seasonal scale from February 2008 to November 2009. At each sampling and at each site, water temperature (°C), salinity (psu), dissolved oxygen (mg l^{-1}) and pH were measured, *in situ*, using a YSI 556 MPS multi-probe. Water samples were taken in triplicate at a depth of 30-50 cm, kept refrigerated and transported to the laboratories as soon as possible for analyses of dissolved nutrients (Table 6). Three sediment replicates for benthos were randomly collected by an Ekman grab (0.0225 m^2 sampling surface), sieved in situ through a 1 mm mesh screen and fixed in 4% buffered formalin. For laboratory procedures see previous section.

2.3 Data analysis

2.3.1 Spatial and temporal analysis

For this investigation, four multimetric indices were selected: BAT (Benthic Assessment Tool; Marques et al., 2009; Teixeira et al., 2009), BITS (Benthic Index based on Taxonomic Sufficiency; Mistri and Munari, 2008), M-AMBI (Borja et al., 2004; Muxika et al., 2007), and ISS (Basset et al., 2011). Indices differ in terms of their general approach, being either taxonomic (BAT, BITS, M-AMBI) or non-taxonomic (ISS), and, in the case of the former, in terms of the level of taxonomic resolution, i.e. species (BAT and M-AMBI) or family (BITS).

The data analysis was based on the transformation of most potential sources of natural variation affecting the four assessment tools into categorical variables. The transformation was required for all factors cited in the national and MEDGIG lagoon typologies, as well as for those factors describing discrete habitat patchiness within ecosystems. Salinity and surface area were transformed into categorical variables in accordance with the Venice System (oligohaline = 0.5-5 psu; mesohaline = 5.1-18 psu; polyhaline = 18.1-30 psu; euhaline = > 30 psu) and the Mediterranean lagoon typology set out in Basset et al. (2006) (small < 2.5 km^2; large > 2.5 km^2). There were too few hyperhaline sites (just four, all in Margherita di Savoia, with a mean salinity of 58.21 psu) to be classified as a separate category and they were thus included in the euhaline group. In addition, the following explanatory variables were characterized for each site: lagoon confinement (two levels: choked and restricted) based on lagoon openness (Barbone and Basset, 2010); sediment grain size (two levels: mud and sand); vegetation (three levels: no vegetation, submerged, emerged); season (two levels: spring and fall). Only two habitat variables were maintained as continuous variables: oxygen and depth. The occurrence of any dependency between the two continuous variables was assessed (Pearson product-moment correlation: $t = 0.54$; $df = 99$; p-value = 0.58). Normality of distribution was checked for each variable using Q-Q plots and outliers were identified by visual examination of Cleveland dot-plots, box-plots, and scatter-plots (Zuur et al., 2009).

The influence of each potential source of variation on the assessment tools was quantified at a uni-dimensional level, by using One-Way ANOVA among levels for each variable and assessment tool, and at the multi-dimensional level by using a mixed-model approach.

A general mixed-model approach (LME, Pinheiro and Bates 2000) was used to assess the effects of the above-mentioned environmental predictors on community descriptors (Barbone et al., 2011), because it takes account of spatial autocorrelation of data (random effects) and unequal variance in variance-covariate terms. As a first step, five general linear regression models were fitted with all the explanatory variables considered. The initial full model is:

Y = salinity*surface + log(depth)*sediment*vegetation + season + oxygen + confinement

where an asterisk between factors indicates their sum and the interaction between them. The validation procedures for the five models (Q-Q plots; residuals versus fitted values) showed evidence of non-spatial independency of data (residual distributions among transitional water bodies) and unequal variance among the explanatory variables. Consequently (Zuur et al., 2009), five mixed models, which incorporate transitional waters as a random effect and the interaction between surface area and salinity as a variance-covariate structure for explanatory variables, were independently developed. The mixed models were refined by manual backward stepwise selection using maximum likelihood (ML) to remove insignificant terms. The importance of independent variables was evaluated by a likelihood ratio test, consisting of a comparison of the full minimal adequate model with models in which the independent variable and all the interaction terms it was involved with were omitted. We set the lower limit of significance at 5%. The coefficients of the final optimal LME were calculated using restricted maximum likelihood (REML). The resulting optimal models (M1-M5) were validated in order to verify that the underlying statistical assumptions were not violated. The normality of residuals was assessed by plotting theoretical quantiles against standardized residuals (Q-Q plots) and homogeneity of variance was evaluated by plotting residual against fitted values. These statistical analyses were conducted using the 'nlme' package (Pinhero et al., 2006) within the 'R' statistical and programming environment (R Development Core Team, 2006). The results of the mixed effect model were also used to define typology *a posteriori,* at the metric-specific level.

The effectiveness of the different *a priori* and *a posteriori* approaches to lagoon typology was tested using One-Way ANOVA, in order to quantify the deviance of the whole sample explained by the selected types. The percentages of deviance explained by each typology were then compared.

Analysis of the Lesina lagoon data focused on the intra-site variability along a seasonal time axis. Two-Way ANOVA, with time (8 levels: fixed) and site (6 levels: fixed) as factors, was used to test for temporal and spatial variation within each multimetric assessment tool. Data were checked for normality using the Kolmogorov-Smirnov test and homogeneity of variances was assessed by Cochran's test. Data were transformed whenever the homoscedasticity assumption was not met.

2.3.2 Ecological Classification

Classification of ecological status was performed by two different procedures: (i) using the standard published boundaries for each multimetric assessment tool; and, (ii) defining new boundaries at the level of lagoon type for each multimetric assessment tool with reference to the distribution of values observed in the sample of reference sites available in the dataset for each type (type-specific boundaries).

As regards the former, the ecological classification of the 101 reference sites was carried out in accordance with the standard boundaries available in literature for M-AMBI (Borja et al., 2004), BAT (Texeira et al., 2009), BITS (Mistri and Munari, 2008) and ISS (Basset et al., 2011).

As regards the latter, the new type-specific boundaries were refined for each type on the same reference sites by setting the boundary between high (H) and good (G) at the 50th percentile of the values of each assessment tool, scaling all other boundaries in accordance with the scaling procedures originally used by the authors (Teixeira et al., 2009; Mistri and Munari, 2008; Muxika et al., 2007; Basset et al., 2011). As an example, the new boundary between good and moderate (M) classes is equal to the new high-good boundary multiplied by the ratio between standard good-moderate and high-good boundaries [new G-M=new H-G*(standard G-M/H-G)]. To this aim, the proposed Mediterranean lagoon typologies were slightly refined by grouping water salinity in only two levels (i.e., <30 psu; >30 psu), according to the results of the mixed model analysis and to account for the small number of sites in the oligohaline and mesohaline types. Therefore, type-specific boundaries were set for 8 types based on the extended Italian typology (2 salinity levels, two surface area levels, two tidal range levels) and for 4 types based on the MEDGIG typology (2 salinity levels and 2 'confinement' levels). In the former, even after this simplification the lack of small lagoons with a tidal range higher than 0.5 m in the dataset had prevented the definition of the new boundaries for 2 out of the 8 Types. New type-specific and metric-specific classification boundaries were also defined from the mixed effect model results. The sites were divided into 8 types when classification was performed with BAT and M-AMBI (2 salinity levels, 2 surface area levels, 2 seasonal levels), into 12 types when classification was performed with BITS (2 salinity levels, 3 vegetation type levels, 2 confinement levels) and into 4 types with a standardisation along the continuous variable oxygen content when the classification was performed with ISS (2 salinity levels, 2 seasonal levels).

The accuracy of the two classification procedures was then tested within and among lagoon typologies by comparing the distribution of sites in the five ecological status quality groups (Contingency analysis; Chi square test).

2.3.3. Validation procedures

The same data analyses described in the above paragraphs were applied to an independent set of disturbed ecosystems, or disturbed sites within ecosystems where a strong pressure gradients were identified, from the TWReferenceNet database.

Validation was performed both at the study site level and at the lagoon/lagoon area level. The accuracy of the classification of reference and disturbed lagoon conditions was compared with the various methodological approaches so far proposed to define Mediterranean lagoon typology (Contingency analysis; Chi square test).

3 Results

3.1. Patterns of spatial variation

3.1.1. Sources of variation

Overall, all multimetric assessment tools showed significant variability both within and among the studied reference ecosystems. They varied significantly among lagoon ecosystems (ANOVA statistics, $p < 0.01$, for all metrics) and were also significantly affected by the environmental factors considered (Table 7; mixed effect models, Table 8). As a general trend, the multimetric assessment tool values increased with increasing salinity from oligo-haline to euhaline lagoons, increased with increasing lagoon surface area, were higher in spring than in fall and, within lagoons, in habitats with submerged vegetation or no vegetation than in habitats with emerged vegetation (Table 7)

Table 7. Mean BAT, BITS, M-AMBI and ISS values measured at each level of factors. Standard error is shown in brackets.

Factors	BAT	BITS	M-AMBI	ISS
Salinity				
Oligohaline	0.44	0.57	0.44	2.66
	(0.04)	(0.11)	(0.04)	(0.15)
Mesohaline	0.54	1.08	0.55	2.65
	(0.06)	(0.21)	(0.05)	(0.20)
Polyhaline	0.55	1.73	0.61	2.71
	(0.03)	(0.20)	(0.04)	(0.16)
Euhaline	0.62	2.11	0.75	3.37
	(0.03)	(0.13)	(0.03)	(0.14)
Surface area				
Large	0.56	1.63	0.67	3.01
	(0.03)	(0.15)	(0.03)	(0.12)
Small	0.54	1.40	0.58	2.86
	(0.03)	(0.17)	(0.03)	(0.12)
Confinement				
Choked	0.53	1.59	0.59	2.88
	(0.02)	(0.12)	(0.02)	(0.11)
Restricted	0.63	1.34	0.73	3.15
	(0.03)	(0.17)	(0.04)	(0.15)
Sediment granulometry				
Mud	0.55	1.77	0.64	2.92
	(0.02)	(0.15)	(0.02)	(0.10)
Sand	0.55	2.06	0.54	3.06
	(0.04)	(0.18)	(0.04)	(0.23)
Vegetation				
No vegetation	0.55	1.77	0.63	2.97
	(0.02)	(0.15)	(0.04)	(0.13)
Submerged vegetation	0.58	1.52	0.67	3.00
	(0.03)	(0.17)	(0.03)	(0.17)
Emerged vegetation	0.53	1.04	0.57	2.79
	(0.03)	(0.18)	(0.04)	(0.14)
Season				
Fall	0.53	1.48	0.60	2.90
	(0.03)	(0.14)	(0.03)	(0.14)
Spring	0.55	1.53	0.63	2.98
	(0.02)	(0.15)	(0.03)	(0.11)

Variability was higher for BITS than for the other assessment tools; at the ecosystem level, the coefficients of natural variation were between 30% and 35% for BAT, M-AMBI and ISS, while the coefficient of variation of BITS was 65.8%. At the study site level, BAT, M-AMBI and ISS were significantly less variable than BITS (Fratio; $F_{99,99}$= 3.83; $F_{99,99}$= 4.71; $F_{99,99}$= 3.61; respectively).

Table 8. Final Linear Mixed Model results for environmental factors' effects on multimetric indices of macroinvertebrate communities in 14 Mediterranean and Black Sea Lagoons. Only terms that are significant for optimal model are shown. Non-significant factors that were part of significant interactions were not removed. Factors (f = fixed; r = random; v-c = variance-covariance).

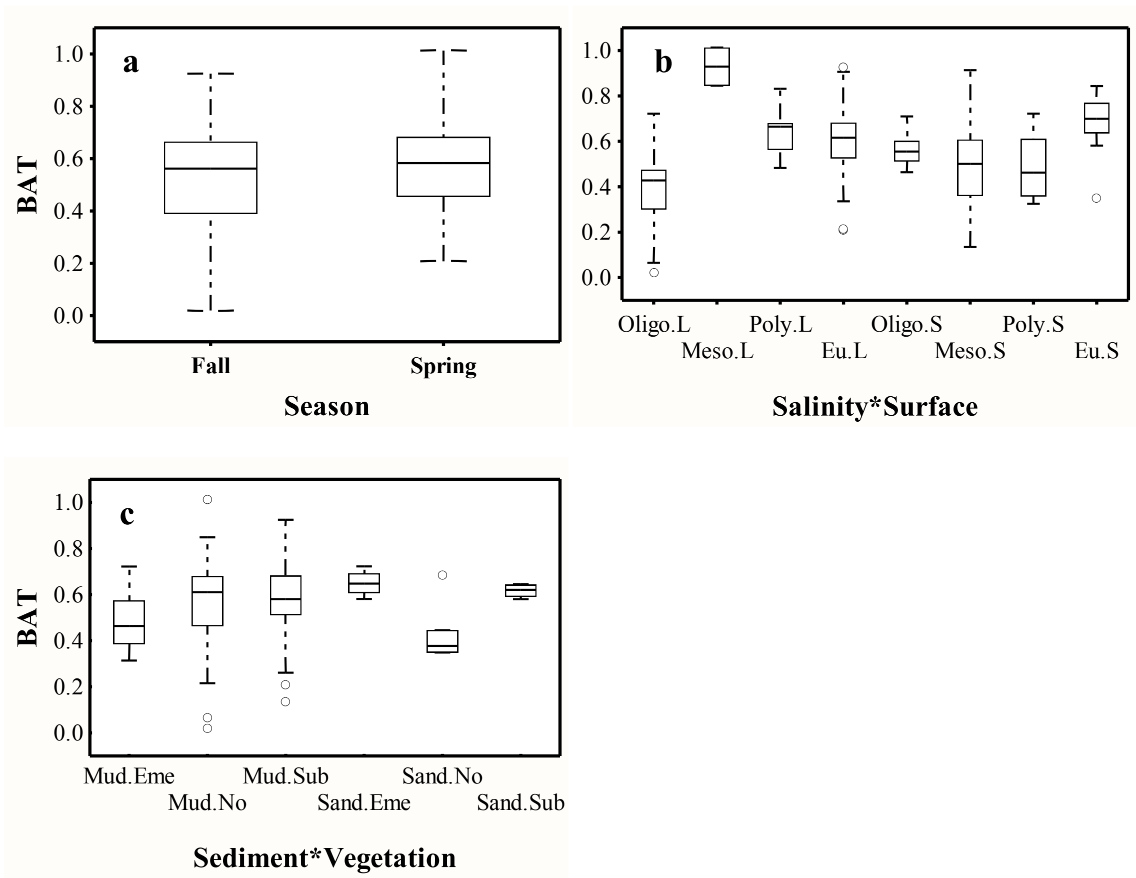
model	response	model terms	f-r	d. f.	l-ratio	*p*
M1	BAT	Salinity	f	3	4.55	0.2075
		Season	f	1	5.29	0.0213
		Surface	f	1	0.42	0.5142
		Salinity*Surface	f	3	19.88	<0.0001
		Sediment	f	1	0.52	0.4691
		Vegetation	f	2	2.10	0.3493
		Sediment*Vegetation	f	2	7.10	0.0287
		Lagoon	*r*	1	12.51	<0.0001
		*Surface*Salinity*	*v-c*	7	9.71	0.2052
M2	BITS	Salinity	f	3	23.11	<0.0001
		Confinement	f	1	11.40	<0.0001
		Vegetation	f	2	21.53	<0.0001
		Season	f	1	20.73	<0.0001
		Lagoon	*r*	1	3.10	0.0391
		*Surface*Salinity*	*v-c*	7	31.43	<0.0001
M3	M-AMBI	Salinity	f	3	9.76	0.0207
		Surface	f	1	1.37	0.2414
		Surface*Salinity	f	3	12.79	0.0051
		Season	f	1	9.31	0.0023
		Lagoon	*r*	1	10.36	0.0013
		*Surface*Salinity*	*v-c*	7	7	0.0350
M4	ISS	Salinity	f	3	9.03	0.0289
		Season	f	1	7.76	0.0053
		Oxygen	f	1	4.61	0.0317
		Lagoon	*r*	1	5.99	0.0144
		*Surface*Salinity*	*v-c*	7	15.58	0.0291

The relevance of the different potential sources of natural variation of the multimetric indices included in Table 7, regarding both typology and intra-lagoon habitat patchiness, was further analysed with an a posteriori approach, using mixed models, at the metric-specific level.

Quantitatively, each multimetric index showed co-variation patterns with various environmental factors (mixed effect models, Table 8). Among the factors considered in the proposed typologies (Table 8): (i) water salinity and seasonality were found to be a significant source of variation for all multimetric indices; (ii) surface area significantly affected BAT and M-AMBI; (iii) confinement and internal sources of variation, such as sediment type or vegetation, were found to be a source of variation of BITS; and (iv) tidal range was not selected as a significant source of variation of multimetric indices.

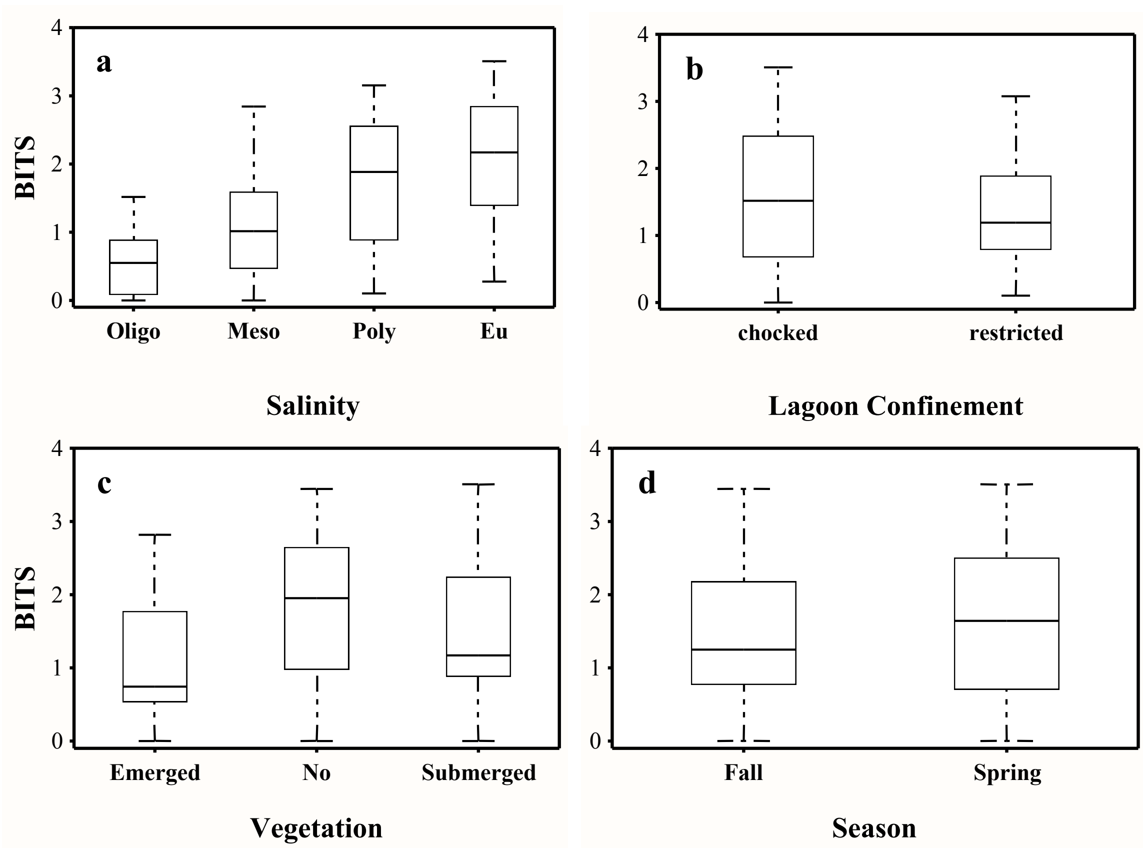
Differences were observed in the environmental factors, which had a significant influence at the multimetric descriptor level. BAT was significantly affected by season, being higher in spring than in fall (L-ratio= 5.29, d.f.= 1, p= 0.0213; Figure 3a) and by the interactions between salinity and surface area (L-ratio = 19.88, d.f. = 7, p< 0.0001; Figure 3b) and between sediment and vegetation (L-ratio= 7.10, d.f.= 5, p= 0.0287; Figure 3c).

Figure 3. Comparisons of mean BAT values under environmental conditions found to be significant sources of variation with mixed modelling statistical approach (MLE). a) sampling season; b) interaction between salinity and surface area (L = large; S = small); c) interaction between sediment and vegetation (Eme = emerged vegetation; Sub = submerged vegetation; no = no vegetation). Central lines represent median value; boxes represent range between lower and upper quartiles (i.e. 25 percent–75 percent); whiskers indicate full range of data, excluding outliers.



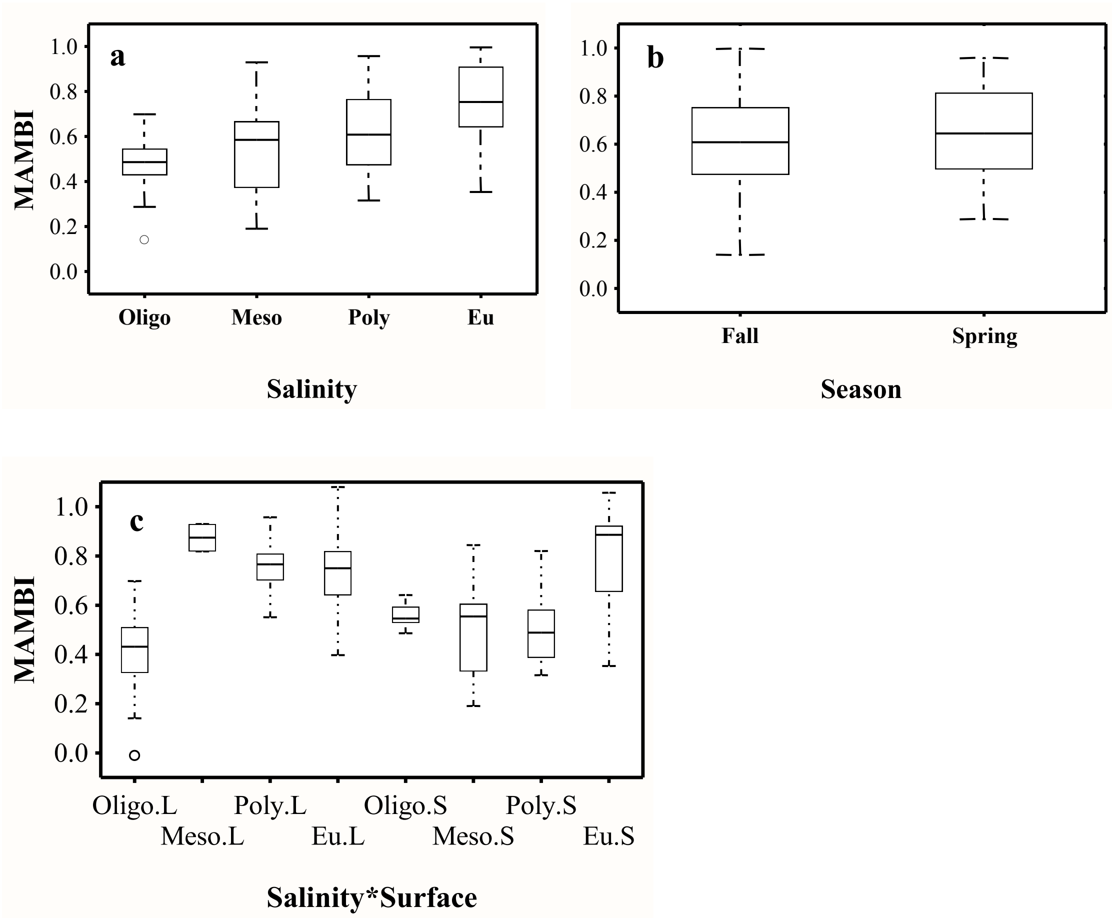
BITS varied significantly as a function of lagoon salinity, lagoon confinement, habitat patchiness and season, being higher in polyhaline and euhaline than oligohaline and mesohaline waters (L-ratio= 23.11, d.f.= 3, p< 0.0001; Figure 4a), in choked than in restricted lagoons (L-ratio= 11.40, d.f.= 1, p< 0.0001; Figure 4b), in the presence of submerged/no vegetation than in patches with emerged vegetation (L-ratio = 21.53, d.f.= 2, p< 0.0001; Figure4c) and in spring than in fall (L-ratio= 20.73, d.f.= 1, p< 0.0001; Figure 4d).

Figure 4. Comparisons of mean BITS values under environmental conditions found to be significant sources of variation with mixed modelling statistical approach (MLE). a) salinity levels (Oligo = oligohaline; Meso = mesohaline; Poly = polyhaline; Eu = euhaline); b) lagoon confinement; c) vegetation type (Emerged vegetation, no vegetation and submerged vegetation); d) sampling season. Central lines represent median value; boxes represent range between lower and upper quartiles (i.e. 25 percent–75 percent); whiskers indicate full range of data, excluding outliers.



M-AMBI was also significantly affected by lagoon salinity and season, being higher in euhaline lagoons than in polyhaline, mesohaline and oligohaline conditions (L-ratio= 9.76, d.f.= 3, p= 0.0207; Figure 5a) and in spring than in fall (L-ratio= 9.31, d.f.= 1, p= 0.0023; Figure 5b). It was also influenced by the interaction between salinity and surface area (L-ratio= 12.79, d.f.= 7, p= 0.0051; Figure 5c).

Figure 5. Comparisons of mean M-AMBI values under environmental conditions found to be significant sources of variation with mixed modeling statistical approach (MLE). a) salinity levels (Oligo = oligohaline; Meso = mesohaline; Poly = polyhaline; Eu = euhaline); b) season; c) interaction between salinity and surface area (L = large; S = small). Central lines represent median value; boxes represent range between lower and upper quartiles (**i.e.** 25 percent–75 percent); whiskers indicate full range of data, excluding outliers.

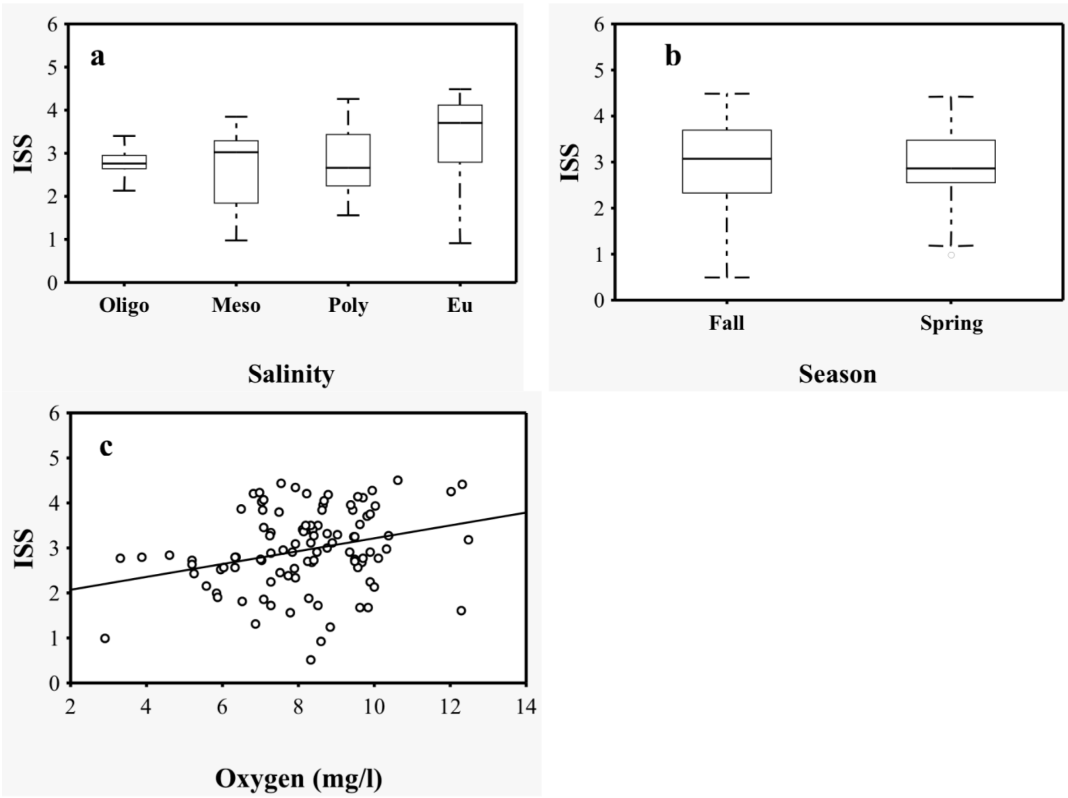


ISS was significantly influenced by salinity levels (L-ratio= 9.03, d.f.= 3, p= 0.0289; Figure 6a), being higher in euhaline than in polyhaline, mesohaline and oligohaline conditions, and by season (L-ratio= 7.76, d.f.= 1, p= 0.0053; Figure 6b), being higher in spring than in fall. ISS increased with oxygen concentration (L-ratio= 4.61, d.f.= 1, p= 0.0317; Figure 6c).

When compared with the single-type “Coastal Lagoons” approach, all more complex typology schemes significantly reduced the variance of the assessment tools (One-Way ANOVA, p< 0.01

for all typology schemes and assessment tools). On average, the typology schemes explained 21.4% of the assessment tools' deviance among sites; the MEDGIG typology explained 19.2%, the mixed model-based typology 20.2% and the more complex extended Italian typology 23.3%. At the assessment tool level, average explained deviance was highest for M-AMBI (29.7%) and lowest for BAT (14.6%).

*Figure 6. Comparison of mean ISS values under environmental conditions found to be significant sources of variation with mixed modeling statistical approach (MLE). a) salinity levels (Oligo = oligohaline; Meso = mesohaline; Poly = polyhaline; EU = euhaline); b) sampling season; c) oxygen concentration. Regression equation ISS=0.143*oxy+1.79. Central lines represent median value; boxes represent range between lower and upper quartiles (i.e. 25 percent–75 percent); whiskers indicate full range of data, excluding outliers.*



3.1.2. Ecological classification

The type a-specific classification boundaries for the assessment of lagoon ecological status with the selected multimetric tools are reported in Tables 9 and 10 as '*standard boundaries*'. The same tables report also the type specific boundaries between ecological status classes computed according to the procedures described in section 2.3.2 for lagoon typologies listed in Table 3 (i.e. Type A – 'Coastal Lagoons'; Type B1 – MEDGIG proposal; Type B2 – Italy extended typology proposal; Type C – *a posteriori* metric-specific proposal) (Tables 9 and 10; '*new boundaries*').

Table 9. Standard and new boundaries of proposed typologies. A – Single-type “Coastal Lagoons”; B1 – MEDGIG proposal; B2 – Italian extended Typology. Standard boundaries apply to all typologies and for Type B1 and B2 only new boundaries are reported.

	A		B1				B2								
			<30‰		>30‰		<30‰ Large		<30‰ Small		>30‰ Large		>30‰ Small		
	Standard Boundaries	New Boundaries	Choked	Restricted	Choked	Restricted	<0.5	>0.5	<0.5	>0.5	<0.5	>0.5	<0.5	>0.5	
BAT															
High/Good	0.8	0.6	0.5	0.6	0.6	0.7	0.5	0.7	0.5		0.6	0.7	0.7		
Moderate/Good	0.6	0.4	0.4	0.4	0.5	0.5	0.4	0.5	0.4		0.5	0.5	0.5		
Poor/Moderate	0.4	0.3	0.3	0.3	0.3	0.3	0.2	0.3	0.3		0.3	0.3	0.3		
Bad/Poor	0.3	0.2	0.2	0.2	0.2	0.3	0.2	0.2	0.2		0.2	0.3	0.3		
BITS															
High/Good	1.8	1.4	1.1	0.8	2.2	1.5	0.6	1.2	1.2		2.1	2.1	1.9		
Moderate/Good	1.4	1.1	0.8	0.6	1.7	1.2	0.4	0.9	0.9		1.7	1.6	1.5		
Poor/Moderate	0.9	0.7	0.5	0.4	1.1	0.8	0.3	0.6	0.6		1.1	1.0	0.9		
Bad/Poor	0.5	0.4	0.3	0.2	0.6	0.4	0.2	0.3	0.3		0.6	0.6	0.5		
M-AMBI															
High/Good	0.8	0.6	0.5	0.6	0.7	0.8	0.5	0.8	0.5		0.8	0.8	0.9		
Moderate/Good	0.6	0.5	0.4	0.4	0.5	0.6	0.3	0.6	0.4		0.6	0.6	0.7		
Poor/Moderate	0.4	0.3	0.3	0.3	0.3	0.4	0.2	0.4	0.3		0.4	0.4	0.4		
Bad/Poor	0.2	0.2	0.1	0.1	0.2	0.2	0.1	0.2	0.1		0.2	0.2	0.2		
ISS															
High/Good	4.0	2.9	2.8	2.7	3.7	3.4	2.7	2.2	2.9		3.6	2.5	3.0		
Moderate/Good	2.8	2.0	1.9	1.9	2.6	2.4	1.9	1.6	2.0		2.5	1.8	2.1		
Poor/Moderate	2.2	1.6	1.5	1.5	2.1	1.9	1.5	1.2	1.6		2.0	1.4	1.7		
Bad/Poor	1.2	0.9	0.8	0.8	1.1	1.0	0.8	0.7	0.9		1.1	0.8	0.9		

Table 10. Standard boundaries and type/metric-specific boundaries for four multimetric indices considered.

	Standard Boundaries	<30‰ Large		<30‰ Small		>30‰ Large		>30‰ Small	
		Fall	Spring	Fall	Spring	Fall	Spring	Fall	Spring
BAT									
High/Good	0.8	0.5	0.6	0.4	0.5	0.6	0.6	0.7	0.7
Moderate/Good	0.6	0.4	0.4	0.3	0.4	0.5	0.5	0.5	0.6
Poor/Moderate	0.4	0.3	0.3	0.2	0.3	0.3	0.3	0.4	0.4
Bad/Poor	0.3	0.2	0.2	0.1	0.2	0.2	0.2	0.2	0.3

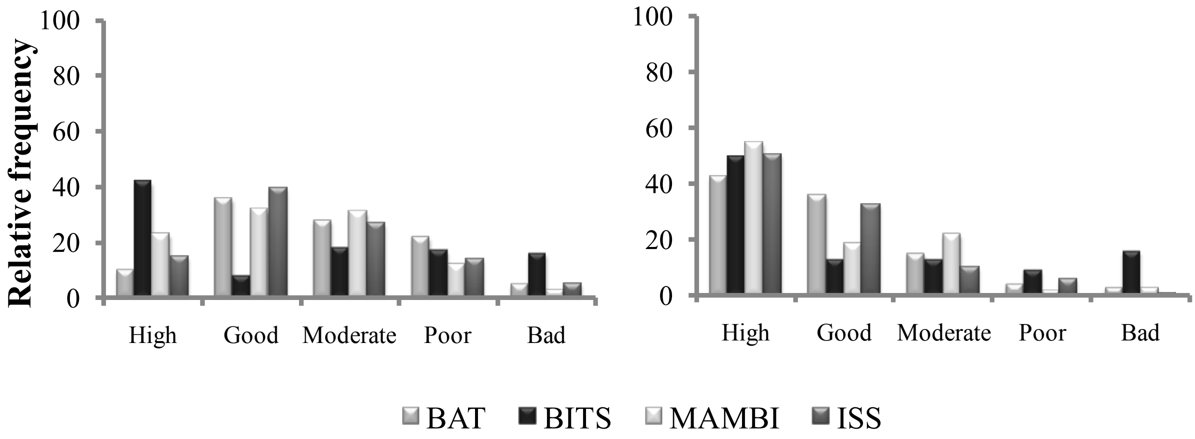
	Standard Boundaries	Choked Fall			Choked Spring			Restricted Fall			Restricted Spring		
		Emerged	No	Submerged	Emerged	No	Submerged	Emerged	No	Submerged	Emerged	No	Submerged
BITS													
High/Good	1.8	1.1	2.2	1.2	0.8	2.5	1.2	0.1	1.0	0.9	0.7	1.8	2.3
Moderate/Good	1.4	0.9	1.7	0.9	0.6	2.0	0.9	0.1	0.8	0.7	0.5	1.4	1.8
Poor/Moderate	0.9	0.6	1.1	0.6	0.4	1.3	0.6	0.1	0.5	0.5	0.3	0.9	1.1
Bad/Poor	0.5	0.3	0.6	0.3	0.2	0.7	0.3	0.0	0.3	0.3	0.2	0.5	0.6

	Standard Boundaries	<30‰ Large		<30‰ Small		>30‰ Large		>30‰ Small	
		Fall	Spring	Fall	Spring	Fall	Spring	Fall	Spring
M-AMBI									
High/Good	0.8	0.5	0.7	0.5	0.5	0.7	0.8	0.7	0.9
Moderate/Good	0.6	0.4	0.5	0.4	0.4	0.5	0.6	0.5	0.7
Poor/Moderate	0.4	0.3	0.4	0.2	0.3	0.4	0.4	0.3	0.5
Bad/Poor	0.2	0.1	0.2	0.1	0.1	0.2	0.2	0.2	0.2

	Standard Boundaries	<30‰		>30‰	
		Fall	Spring	Fall	Spring
ISS					
High/Good	4.0	2.6	2.8	3.4	3.5
Moderate/Good	2.8	1.8	2.0	2.4	2.4
Poor/Moderate	2.2	1.4	1.5	1.9	1.9
Bad/Poor	1.2	0.8	0.9	1.0	1.0

When a single type 'Coastal Lagoons' was considered (Table 3; Type A), the classification using the standard published boundaries strongly underestimated the ecological status of the reference sites (Table 9; Figure 7). ISS, and M-AMBI classified 55.5% of the 'reference' sites as having high or good status, BITS 47.5% and BAT 45.5%. The accuracy of the ecological status classification was significantly improved by using the new boundaries, which were set in this study (Contingency analysis: BAT - $\chi^2 = 37.44$, d.f.= 4, p< 0.01; BITS - $\chi^2 = 5.15$, d.f.= 4 p= n.s; M-AMBI - $\chi^2 = 24.37$, d.f.= 4, p< 0.01; ISS - $\chi^2 = 40.34$, d.f.= 4, p< 0.01); on average 74.3% of sites were classified good or high, with a range of 62.4% (BITS) to 83.2% (ISS).

Figure 7. Classification a priori, based on Greek lagoon typology, of ecological quality status of 101 sites in terms of BAT, BITS, M-AMBI and ISS with standard (a) and type-specific boundaries (b).



Similar results were observed for the other proposed typologies as well as for the typology derived *a posteriori* using the mixed model approach. With all typologies, the new type-specific and metric-specific boundaries significantly improved the accuracy of the ecological status assessment for the studied lagoons (Figures 8 to 10; Contingency analysis: all cases p< 0.05). According to BAT, the reference sites classified as good and high increased from 45.5% to 74.3% (Table 3; Type B1), to 73.3% (Table 3; Type B2) and to 80.2% (Table 3; Type C), they increased from 55.5% to 84.2%, 78.2% and 77.2%, respectively, according to M-AMBI, from 55.5% to 81.2%, 84.2% and 85.1% according to ISS and from 47.5% to 66.3%, 63.4% and 63.4% according to BITS. On average, the ecological status was underestimated at more than 25% of the reference sites (Table 11).

The average accuracy of the ecological status assessment was not affected by the typologies tested in this paper (Contingency analysis; p> 0.05 for all multimetric indices).

Figure 8. Classification a priori, based on lagoon typology proposed from MEDGIG, of ecological quality status of 101 sites ***in terms of*** *BAT, BITS, M-AMBI and ISS with type-specific boundaries.*

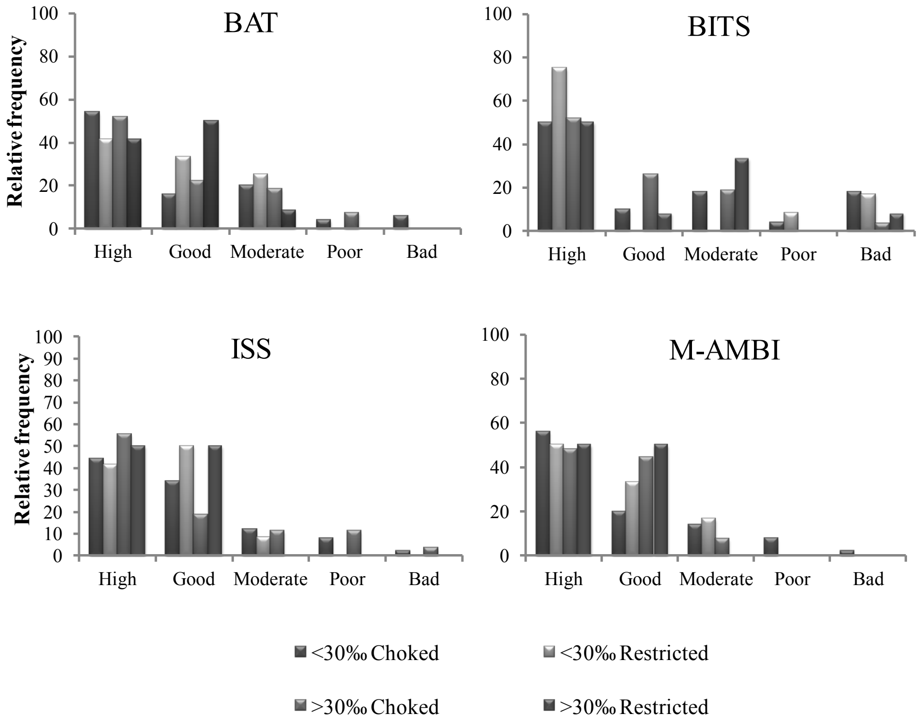


Figure 9. Classification a priori, based on Italian lagoon typology, of ecological quality status of 101 sites ***in terms of*** *BAT, BITS, M-AMBI and ISS with type-specific boundaries. Surface area levels (L = large; S = small).*

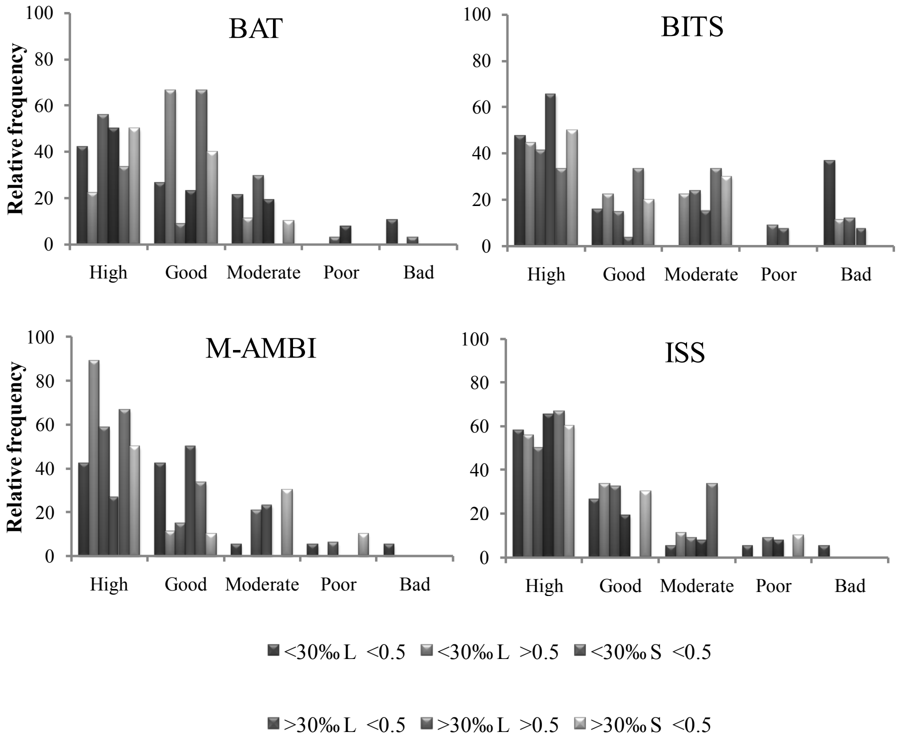


*Figure 10.Classification a posteriori, based on results of mixed models, of ecological quality status of 101 sites **in terms of** BAT, BITS, M-AMBI and ISS with type-specific and metric-specific boundaries. Surface area levels (L = large; S = small); season levels (F = fall; S = spring); confinement levels (C = choked R = restricted); Vegetation type levels (EME = Emerged vegetation, NO = no vegetation and SUB = submerged vegetation).*

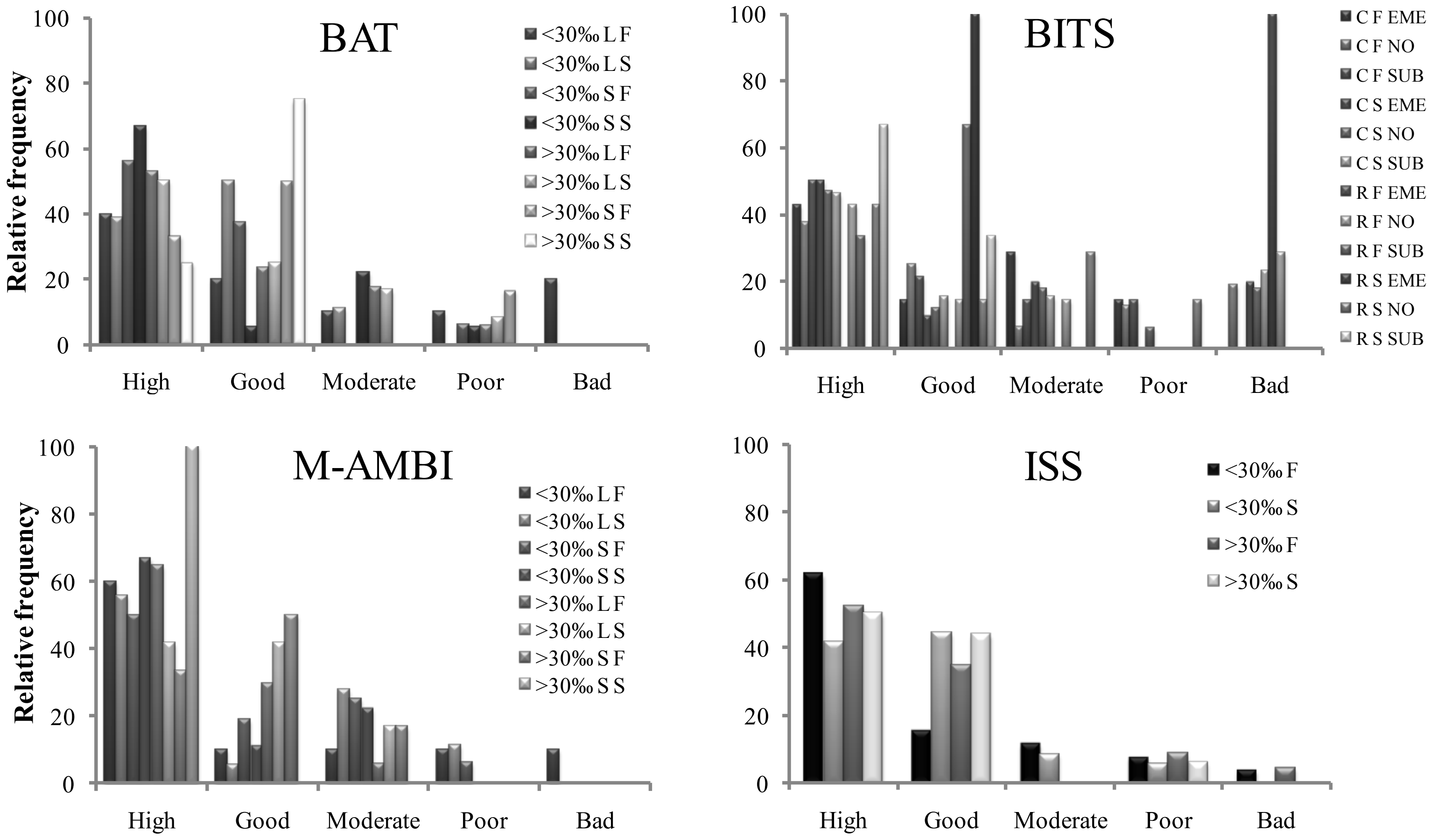


Table 11. Partitioning of reference condition study sites at boundary between good and moderate ecological status for different typologies and multimetric indices.

		Multimetric indices			
		BAT	BITS	M-AMBI	ISS
Type A	High/Good	79	63	84	74
	Moderate/Bad	22	38	17	27
Type B1	High/Good	75	67	85	82
	Moderate/Bad	26	34	16	19
Type B2	High/Good	74	64	79	85
	Moderate/Bad	27	37	22	16
Type C	High/Good	81	64	78	86
	Moderate/Bad	20	37	23	15

3.1.3 Validation procedures

The accuracy of the new type-specific reference condition boundaries was validated using an independent set of data on six lagoons, or lagoons areas, which were known to be affected by strong anthropogenic pressures, using ISS as an assessment tool. The data referred to a total of 57 new study sites.

At the study site level, the accuracy of the ecological status classification of disturbed sites was higher with the standard boundaries than with the type-specific boundaries developed in this study. Only 19.3% of the disturbed sites were classified as having good or high status using the standard boundaries, while on average 52.6% of sites were classified as good or high status using the new type-specific boundaries (Table 12).

Table 12. Percentage of disturbed sites with "Bad", "Poor", "Moderate", "Good" and "High" Ecological Quality status for each proposed typology, considering ISS as assessment tool.

	Type A		**Type B1**	**Type B2**	**Type C**
	standard	*new*			
High	0.00	14.04	10.53	7.02	8.77
Good	19.30	42.11	42.11	42.11	43.86
Moderate	22.81	17.54	14.04	12.28	14.04
Poor	49.12	17.54	24.56	29.82	24.56
Bad	8.77	8.77	8.77	8.77	8.77

Averaging the study site values at the lagoon level, the accuracy of ecological status classification was higher than at the study site level. When the new boundaries were set, all reference lagoons or lagoon areas were classified as good or high with the Type B2 and C approaches and 92.9% with the Type A approach (Table 13). However, while the latter approach classified all disturbed lagoons or lagoon areas as having at least good ecological status, the B1 and B2 approaches classified 83.3% of them as moderate (Table 13)

Table 13. Ecological status of reference lagoons/lagoon areas and disturbed lagoons/lagoon areas for each proposed typology.

		Type A		Type B1	Type B2	Type C
		standard	*new*			
Reference lagoons/lagoon area	*Agi*	Moderate	Moderate	Moderate	Good	Good
	Ali	Good	Good	Good	Good	Good
	Ces	Good	High	High	High	High
	GM	Moderate	Good	Good	High	High
	GVC	Moderate	Good	Good	Good	Good
	GVP	Moderate	Good	Good	Good	Good
	Kar	High	High	High	High	High
	Lea	Moderate	Good	Good	High	High
	Log	Moderate	Good	Moderate	Good	Good
	MdS	Good	High	Good	Good	Good
	Narta	Good	High	Good	Good	Good
	Patok	High	High	High	High	High
	Sinoe	Moderate	Good	Good	Good	Good
	TG	Moderate	Good	Good	Good	Good
Disturbed	*GM*	Poor	Moderate	Good	Good	Good
	GVP	Moderate	Moderate	Moderate	Moderate	Good
	Log	Poor	Moderate	Moderate	Moderate	Moderate
	MdS	Moderate	Good	Moderate	Moderate	Moderate
	Narta	Moderate	Moderate	Moderate	Moderate	Moderate
	Varna	Poor	Moderate	Moderate	Moderate	Good

3.2. Patterns of temporal variation

3.2.1. Sources of variation

The temporal variability of the multimetric indices and the resulting ecological status classification was evaluated at the site/station-specific level in Lesina lagoon. The multimetric index values varied at the site/station level with time. The average coefficients of variation of BAT, ISS and M-AMBI ranged between 18% and 21%, while BITS showed a much higher average variability (39.1%). The values also varied among sites/stations at each sampling time, with average coefficients of variation between 18% and 43%. These observed differences among times and sites were highly significant (two-way Anova, Table 14).

Table 14. Results of two-way analysis of variance among times and stations for four selected metrics in Lesina lagoon.

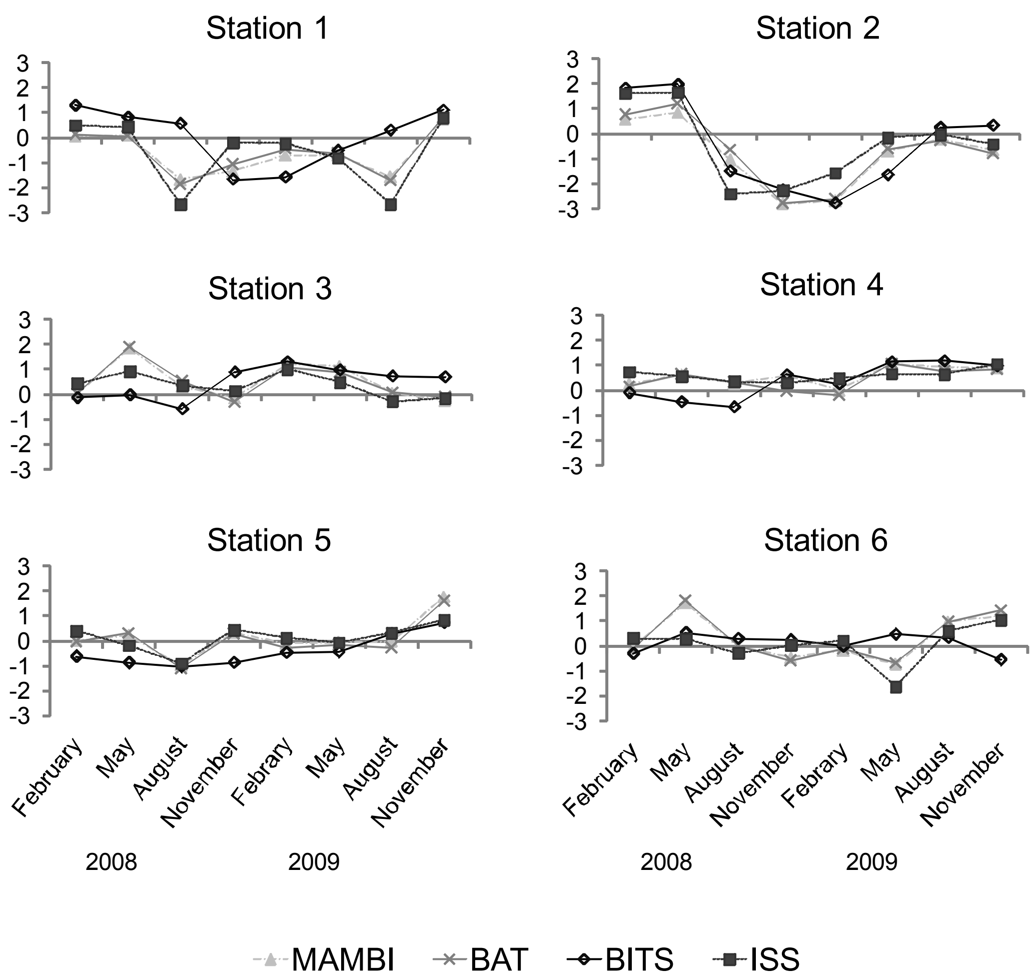
2-way ANOVA	Source	SS	df	MS	F	p
BAT	Time	0.23	7	0.03	10.44	***
	Station	0.19	5	0.04	12.40	***
	Time*Station	0.37	35	0.01	3.35	***
	Error	0.30	96	0.00		
BITS	Time	2.84	7	0.41	25.43	***
	Station	1.46	5	0.29	18.32	***
	Time*Station	9.56	35	0.27	17.09	***
	Error	1.53	96	0.02		
M-AMBI	Time	0.20	7	0.03	11.10	***
	Station	0.24	5	0.05	18.45	***
	Time*Station	0.34	35	0.01	3.78	***
	Error	0.25	96	0.00		
ISS	Time	1.90	7	0.27	9.68	***
	Station	1.28	5	0.26	9.16	***
	Time*Station	3.71	35	0.11	3.79	***
	Error	2.69	96	0.03		

The differences among stations are due to substantially lower abundance, richness and sensitivity values at stations 1 and 2, which were classified as disturbed on the basis of the external pressure values shown in Table 4. The other stations were classified as undisturbed (Table 4). All multimetic indices presented significantly lower values at stations 1 and 2 than the others (Figure 11).

Regarding the temporal component, all multimetric indices showed similar patterns at each station (Figure 11). Average similarity among multimetric indices ranged between 73.6% at station 1 and 75.5% at station 5. Along the time-scale, consistent seasonal variations in the two considered years were observed only at station 1, where all metrics showed significantly lower values during summer than any other season (ANOVA statistics, $p<0.001$, for all metrics). In station 2, which suffered a dystrophic event, there was a reduction of all metrics values after the dystrophic crisis (ANOVA statistics, $p<0.0001$, for all metrics). In the other stations, used as references, consistent seasonal variation was not observed (Figure 11).

It is interesting to note that there is a correspondence of variation patterns observed between multimetric indices based on different types of data and computation method. A correspondence was observed at all stations, being much stronger at stations 1 and 2, which were affected by anthropogenic pressures.

Figure 11. Temporal pattern of standardized multimetric indices in Lesina lagoon sampling stations.



4. Discussion

It is well known that the classification of the ecological status in Mediterranean lagoons is challenging (Basset, 2010); in fact, on the one side lagoons are ecotone ecosystems (Basset et al., 2011 submitted), naturally enriched and with strong variation gradients, on the other side lagoon colonisers show adaptations to these conditions being both resistant and resilient to anthropogenic external pressures. Challenges include distinction of pressures (natural *vs.* anthropogenic), quantification of biological responses and setting of boundaries (Dauvin and Ruellet, 2009; Birk and Hering, 2009), which need to be adjusted when compared to ecosystem categories with lower natural disturbance (Prato et al., 2009). This investigation highlights a number of methodological issues useful to address and overcome the challenge.

Here, we have shown that even under reference conditions an high natural variability of four multimetric indices used or recently proposed as national metrics in different Mediterranean countries can be observed both on the spatial scale, among study sites within lagoons or lagoon areas, and on the temporal scale, among times at a same site.

The results achieved in this study do not seem to depend on the criteria used in this investigation to define reference conditions of Mediterranean lagoons; the criteria used are consistent with the options proposed by the WFD and with the available literature on freshwater and marine ecosystems (Stoddard et al., 2006; Borja et al., 2009a) as well as on a recent study performed on Mediterranean and Black Sea lagoon ecosystems (Barbone et al., 2012). However, since the criteria used are based on minimal disturbance and expert view assessments, which incorporate a certain degree of uncertainty there is still a lack of quantitative definitions of the disturbance level required to consider a site as a reference; it was already acknowledge in the other studies (Lucena-Moya et al., 2009; Neto et al., 2010; Barbone et al., 2012).

Similarly, the results achieved in this study do not seem to depend on the selected transitional water typologies. In fact, in this investigation we have considered all lagoon typologies so far proposed by the Mediterranean countries (i.e. Type A – 'Coastal Lagoons'; Type B1 – MEDGIG proposal; Type B2 – Italy extended typology proposal) as well as a more complex definition of typology (Type C – *a posteriori* metric-specific proposal) to derive type-specific reference conditions that significantly reduce the uncertainty of the assessment in reference condition ecosystems. In this investigation, we have also defined new boundaries for every proposed typology of Mediterranean lagoons. An arguable question concerns the definition of the boundary between the 'Good' status and the 'Moderate' one, namely what is an 'acceptable' (undegraded) or 'not acceptable'(degraded) status or when it is necessary to spend resources for the restoration of an ecosystem (Blanchet et al., 2008). The applicability of multimetric indices in relation to their boundaries of ecological classification was recently analysed in transitional water ecosystems (Dauvin, 2007; Ruellet and Dauvin, 2007), pointing out that sometimes arbitrary boundaries between categories remain (Blanchet et al., 2008).

The approach used in this investigation to define type-specific reference conditions and their boundaries in Mediterranean lagoons, it was been well-developed in freshwater ecosystems with probability tools such as RIVPACS (Wright et al., 1993) and BEAST (Reynoldson et al., 1995), and it uses potential reference sites for biological status evaluation and assesses the level of alteration on the basis of the probability that a test site falls within the range of variation of reference sites. The criterion used in the present report, i.e., setting the boundary between high and good status at the 50th percentile of the distribution of assessment tool values in reference ecosystems is consistent with previous approaches based on type specific reference conditions data. The same criterion was already used for Mediterranean and Black Sea lagoons (Barbone et al., 2012) and a consistent criterion was applied at freshwater ecosystems by the RIVPACS approach, which had generally considered as reference the sites falling within the central 80% of reference spectra; more restrictive criteria, with the H-G boundary set at percentiles equal or higher then 90th, were used for transitional and coastal water ecosystems when the original data were from ecosystems covering a range of disturbance conditions (Borja and Tunberg, 2011).

In this study, we showed that the lagoon typologies, adopted or proposed at the national or MEDGIG level, actually incorporate the key sources of macroinvertebrate assessment tools variability. In fact, all multimetric indices showed patterns of variations with water salinity (Boix et al., 2005; Lucena-Moya et al., 2009; Barbone et al., 2012), which is a main component of the Venice system and of the confinement theory (Guelorget and Perthuisot, 1983). Moreover, BAT and M-AMBI showed patterns of variation with the surface area (Basset et al., 2006) and BITS was affected by confinement.

As regards lagoon salinity, different hypothesis can be considered to explain the patterns of variation shown by all multimetric indices, i.e., dividing euhaline sites from poly- to oligohaline ones characterised by lower values of the assessment tools. Since the nutrient input in lagoon ecosystems are mainly through the freshwater, these differences on the discrete gradient of salinity, with lower values of all assessment tools at meso- to oligohaline sites than at euhaline ones, might be attributable to processes of natural eutrophication at low salinity lagoons or lagoon areas. In fact, an inverse relationship between increasing eutrophication and BAT (Neto et al., 2009 or Texeira et al., 2009), BITS (Munari et al., 2010), M-AMBI (Borja et al., 2009) and ISS (Basset et al., 2012) values have already been showed. Clearly, stream and rivers can also transport into the lagoon ecosystems organic and inorganic pollutants, but it seems to be an unlikely explanation of the patterns observed in the reference lagoons, since they were affected by low-pressure intensity. On the other hand, since all indices are someway affected by species richness and most lagoon species are marine in origin, the higher values of all assessment tools at euhaline sites might be attributable to a stronger influence of the sea and to a higher colonisation rate from marine species (Whitfield et al., in press).

The variation of BAT and M-AMBI with lagoon surface area is also consistent with the existing literature. In fact both of them have a taxonomic richness component and the occurrence of well-defined species-area relationships have already been observed for Mediterranean lagoons (Sabetta et al., 2007). This means that clear reference conditions for these indices, regarding the sampling size area, must be defined, as stressed by some authors (Borja et al., 2004, 2012).

On the other hand, the fact that the multimetric indices were not affected by the internal lagoon heterogeneity, with the exception of BITS, suggests that the integration of simple metrics increases the resistance of the assessment tools to the spatial patchiness of lagoon ecosystems. In fact simple metrics were observed to be sensitive to patchiness in vegetation, sediment types or both (Barbone et al., 2012). The sensitivity of BITS to the vegetation habitat type has already been showed for Mediterranean lagoons (Munari and Mistri, 2010). These results highlight that some heterogeneity in the habitat type sampled at different spatial scales (lagoon, regional area, eco-regional area) should not introduce significant biases in the evaluation process of lagoon ecological status.

The results of this report also highlight the important influence of the seasonal patterns on all assessment tools tested here on macroinvertebrate guilds. It is supported by the fact that all assessment tools showed significantly higher values in spring than in fall. These patterns are likely to be related to both abiotic patterns of temperature and dissolved oxygen characterising Mediterranean lagoons and on species responses with life cycle adaptation and space use behaviour. In fact, summer crisis are common in Mediterranean lagoons not only as a result of external inputs of nutrient from anthropogenic activities (Zaldivar et al., 2008) but also depending on reduced hydrodynamics and wind-driven mixing (Vignes et al., 2009). This pattern is more evident in Lesina lagoon, where all multimetric indices respond on anthropogenic disturbance event such as a dystrophic crisis with a pronounced reduction of all values after the dystrophic crisis coming. In fact the ecological quality status for the station 2, affected by the crisis, changed from “good” or “moderate” ecological condition to “poor” or even “bad” condition. This temporal variability must have to be taken into account both to standardise the time schedule in the monitoring programs to assess the ecological status of Mediterranean lagoons and to derive season specific reference conditions.

In this investigation, we have classified the sites inside the lagoons using for each multimetric assessment tool both standard published boundaries and new boundaries defined considering the Mediterranean lagoon typologies proposed and the more complex typology obtained with the results of mixed effect models. This approach is similar to that used in the European intercalibration exercise (Borja et al., 2007, 2009b).

The classification with the standard boundaries underestimated the ecological status of the reference sites considered, 50% of which were on average classified as moderate, poor or bad. The level of mis-classification was independent of the multimetric indices selected. Consequently, all the indices tested appear to be potentially appropriate to classify ecological status of reference lagoons while their boundaries between the ecological quality classes were clearly not adequate.

The fact that mis-classification was not completely removed by setting the new type-specific and metric-specific boundaries, which anyway significantly improved the accuracy of every multimetric index, is attributable to different factors. Hidden anthropogenic pressures might occurring on some lagoon area for lateral or aerial diffusion, or historical anthropogenic pressures stored and slowly released from the sediments might have determined the macroinvertebrate response (Barbone et al., 2012). Moreover, natural pressures are likely to have different intensity at different sites within any fixed lagoons due to patchy sedimentation rates, spatially explicit gradients of hydrodynamic forces, differential influence of freshwater and marine waters, locally determining underestimates of the real ecological status of sites within lagoon ecosystems or lagoons within lagoon complexes. The occurrence of mis-classification was significantly lower when new rather than standard boundaries were used, but still significant. Using type and metric specific reference conditions for the assessment tools considered, part of sites (25%) were understestimated, showing a potential bias in the classification of reference lagoon ecosystems still occur in our data set and that the low values of macroinvertebrate assessment metrics are not necessarily a index of underestimation of the actual ecological status of lagoon ecosystems.

The potential misclassification also observed on the validation data set, when only ISS was used as an assessment tool, suggested an alternative explanation based on the adaptation of benthic macroinvertebrates to very harsh and variable conditions. Macroinvertebrates are likely to be highly resistant and resilient, other than pre-adapted, to disturbed conditions.

The risk of performing a potential bias in the classification of the ecological status is in some way intrisically related to the particular conditions of Mediterranean lagoons, naturally eutrophic and highly variable ecosystems. Consequently, a strong effort is actually required from applied aquatic research to fully evaluate this risk and develop improved procedure to minimise it.

5. Conclusions

Our study shows that:

1. multimetric assessment tools of benthic macroinvertebrates have a large variability within and among reference lagoon ecosystems in the Mediterranean and Black Sea Ecoregions;
2. the multimetric assessment tools are generally robust to potential heterogeneity sources determined by habitat patchiness within lagoons, while they are significantly affected by abiotic drivers of among lagoon heterogeneity;
3. the different approaches to typology, adopted or proposed at the national or MEDGIG level, actually incorporate the key abiotic drivers of the assessment tool variability, even though the amount of variation explained can be low in some cases;
4. a relevant influence of the seasonal period, not included in any typological scheme, on all assessment tools is emphasised;
5. the development of new type specific classification boundaries significantly improve the assessment accuracy of the four multimetric indices on reference lagoons or lagoon areas;
6. a potential misclassification, suggesting an over-estimation of the ecological status, is observed at the study site level in the validation test when the new type-specific boundaries are applied at anthropogenically disturbed lagoon ecosystems or lagoon areas;

7. a significant variability in ecological status assessment, both at disturbed and at undisturbed sites, was also observed on a temporal scale for each multimetric descriptor tested for the assessment; nevertheless, on average all indices differentiated the disturbed from the undisturbed sites;
8. on average, all multimetric indices were able to discriminate reference and disturbed lagoon or lagoon areas when the new type specific boundaries derived in this deliverable from a group of reference sites were applied at the ecosystem level; on the other hand, the use of the standard type aspecific boundaries available in the literature determines strong under-estimates of the ecological status of lagoon ecosystems.

The validation test showed that in lagoon ecosystems when dealing with benthic macro-invertebrates the actual functional distance between neighbouring ecological status is likely to be very short; alternatively, macroinvertebrates, which fully experience the sharp ecological gradients characterising lagoon ecosystems are pre-adapted to disturbed conditions, being both highly resistant and highly resilient. Both explanations acknowledge a degree of uncertainty in the assessment of lagoon ecological status with benthic invertebrates asking for an optimisation of all procedures in order to avoid misclassification. In fact, following the WFD scheme misclassification will cause not required high recovery costs, in case of under-estimates, or strong ecological risks and delayed remediation measures, in case of over-estimation.

In order to minimise the misclassifications or the delay in the recovery actions we highlight the following recommendations for the monitoring programmes on Mediterranean and Black Sea lagoons:

1. Sampling design need to account for the spatial variability and for a minimum number of sites in each lagoon; replicate sites prevent under estimation of the actual ecological status potentially due to the intrinsic variability shown by all assessment tools in lagoon ecosystems;
2. Sampling design need to include seasonal samplings or to refer to type and time specific reference conditions; all assessment tools had values under-estimating ecological status in fall when compared to spring;
3. Each Typology can be used and each assessment tool can be used without large decrease in the assessment accuracy, but the use of type specific reference condition boundaries is required;
4. Classification performed at the lagoon level is more accurate than at the study site level, avoiding also misclassification and not required remediation actions; however, moderate to bad sites within otherwise good ecological status lagoons need surveillance or investigative monitoring schemes until a clear pressure response relationship is quantified at the site level.
5. (integrations are welcome).

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