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WISER

Water bodies in Europe: Integrative Systems to assess Ecological status and Recovery

DELIVERABLE

Deliverable 4.3-1: Manuscript on the responses of existing indicators to different pressures

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Content

Non-technical summary

The structure of the benthic macroinvertebrate fauna is one of the quality elements used in the Water Framework Directive (WFD) to assess ecological quality status in Europe. Several different indices have been proposed and may be used to classify benthic status. In this Deliverable we compared single metrics and multimetric methods to assess coastal and transitional benthic status along human pressure gradients in five distinct environments across Europe: Varna bay (Bulgaria), Lesina lagoon (Italy), Mondego estuary (Portugal), Basque coast (Spain) and Oslofjord (Norway). Hence, 13 single metrics and 8 of the most common indices used within the WFD for benthic assessment were selected. As single metrics, abundance, species richness (as number of taxa), Shannon's diversity, AMBI (AZTI's Marine Biotic Index), five ecological groups (from sensitive to opportunistic species), Margalef index, SN, ES100, and ES50, were calculated. As multimetric or multivariate methods ISS (Index of Size Spectra), BAT (Benthic Assessment Tool), NQI (Norwegian Quality Index), M-AMBI (multivariate AMBI), BQI (Biological Quality Index), BEQI (Benthic Ecosystem Quality Index), BITS (Benthic Index based on Taxonomic Sufficiency), and IQI (Infaunal Quality Index) were calculated. Within each system, sampling sites were ordered in an increasing pressure gradient according to a preliminary classification based on professional judgement, and the response of single metrics and assessment methods to different human pressure levels was evaluated. The different indices are largely consistent in their response to pressure gradient, except in some particular cases (i.e. BITS, in all cases, or ISS when a low number of individuals is present). Inconsistencies between indicator responses were mostly in transitional waters (i.e. IQI, BEQI), highlighting the difficulties of the generic application of indicators to all marine, estuarine and lagoonal environments. However, some of the single (i.e. ecological groups approach, diversity, richness, SN) and multimetric methods (i.e. BAT, M-AMBI, NQI) were able to detect such gradients both in transitional and coastal environments. This study highlights the importance of survey design and good reference conditions for some indicators. The agreement observed between different methodologies and their ability to detect quality trends across distinct environments constitutes a promising result for the implementation of the WFD's monitoring plans.

Title: Response of single benthic metrics and multimetric methods to anthropogenic pressure gradients, in five distinct European coastal and transitional ecosystems

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Abstract

The structure of the benthic macroinvertebrate fauna is one of the quality elements used in the Water Framework Directive (WFD) to assess ecological quality status in Europe. Several different indices have been proposed and may be used to classify benthic status. In this study we compared single metrics and multimetric methods to assess coastal and transitional benthic status along human pressure gradients in five distinct environments across Europe: Varna bay (Bulgaria), Lesina lagoon (Italy), Mondego estuary (Portugal), Basque coast (Spain) and Oslofjord (Norway). Hence, 13 single metrics and 8 of the most common indices used within the WFD for benthic assessment were selected. As single metrics, abundance, species richness (as number of taxa), Shannon's diversity, AMBI (AZTI's Marine Biotic Index), five ecological groups (from sensitive to opportunistic species), Margalef index, SN, ES100, and ES50, were calculated. As multimetric or multivariate methods ISS (Index of Size Spectra), BAT (Benthic Assessment Tool), NQI (Norwegian Quality Index), M-AMBI (multivariate AMBI), BQI (Biological Quality Index), BEQI (Benthic Ecosystem Quality Index), BITS (Benthic Index based on Taxonomic Sufficiency), and IQI (Infaunal Quality Index) were calculated. Within each system, sampling sites were ordered in an increasing pressure gradient according to a preliminary classification based on professional judgement, and the response of single metrics and assessment methods to different human pressure levels was evaluated. The different indices are largely consistent in their response to pressure gradient, except in some particular cases (i.e. BITS, in all cases, or ISS when a low number of individuals is present). Inconsistencies between indicator responses were mostly in transitional waters (i.e. IQI, BEQI), highlighting the difficulties of the generic application of indicators to all marine, estuarine and lagoonal environments. However, some of the single (i.e. ecological groups approach, diversity, richness, SN) and multimetric methods (i.e. BAT, M-AMBI, NQI) were able to detect such gradients both in transitional and coastal environments. This study highlights the importance of survey design and good reference conditions for some indicators. The agreement observed between different methodologies and their ability to detect quality trends across distinct environments constitutes a promising result for the implementation of the WFD's monitoring plans.

Key words

Indices, multimetric methods, benthic fauna, pressure gradient, coastal and transitional waters, Water Framework Directive

1 Introduction

In recent times, legislation worldwide has been seeking suitable methods to assess anthropogenic impacts on marine ecosystems, using different elements of the system (Borja *et al.,* 2008). Benthic macroinvertebrates, as one of these elements, have been used for long time to assess environmental impacts from human pressures (Littler and Murray, 1975; Pearson and Rosenberg, 1978; Dauer, 1993). In this context, over the last decade, a plethora of single and multimetric indices have been developed, mainly in Europe and the USA (Diaz *et al.*, 2004; Pinto *et al.,* 2009).

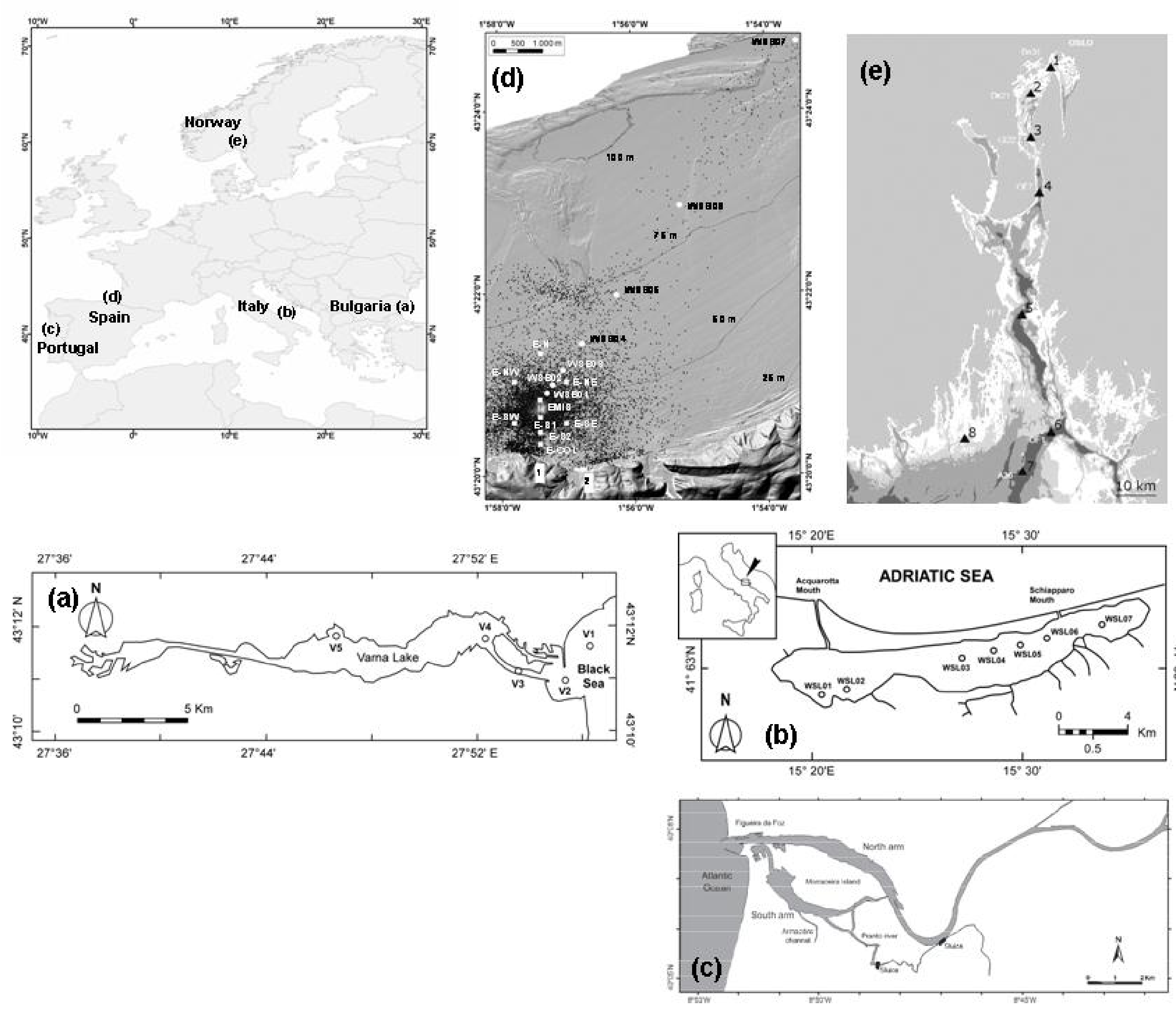
Borja and Dauer (2008) and Borja *et al.* (2009c) have discussed the steps in an index development, which include: (i) selection of candidate metrics; (ii) metric combination; (iii) index validation; (iv) index application to different human pressures; (v) index interpretation; and (vi) index intercalibration. Much of this development has taken place within the European Water Framework Directive (WFD), trying to look for suitable methods to assess the benthic ecological status in marine and estuarine waters (Borja *et al.,* 2009b).

Although much attention has been paid, in the USA and Europe, to the three first steps (e.g. Engle *et al.,* 1994; Weisberg *et al.,* 1997; van Dolah *et al.,* 1999; Borja *et al.,* 2000; Rosenberg *et al.,* 2004), and, to some extent, to the response of the indices to human pressures (i.e. Chainho *et al.,* 2008; Josefson *et al.,* 2009; Borja *et al.,* 2009a; Neto *et al.,* 2010), there have been few intercalibration studies (Borja *et al.,* 2007, 2009b; van Hoey *et al.,* 2007b). Furthermore, the response of the metrics combined into the multimetric indices to different pressure gradients has not been widely investigated (Lavesque *et al.,* 2009).

Within this context, the identification of pressure-response relationships of coastal and transitional (‘transitional’ refers to estuaries and lagoons, after the WFD) benthic invertebrates, using existing metrics and multimetric methods for soft-bottom habitats assessment, can be considered highly useful in the implementation of new legislation.

Hence, the objective of this contribution is to test the capability of several metrics and multimetric indices, to discriminate between different stressors (discharges, port activity, etc.), along human pressure gradients, across a wide range of European marine geographical regions and systems, including both transitional and coastal waters. The locations selected were: Varna bay (Bulgaria), Lesina lagoon (Italy), Mondego estuary (Portugal), Basque coast (Spain), and Oslofjord (Norway) (Figure 1).

Figure 1. Study sites, showing all locations cited within the text and the sampling stations, together with main anthropogenic pressures. (a) Varna bay coast; (b) Lesina lagoon; (c) Mondego estuary; (d) Mompás-Pasaia coast (small black dots indicate particle dispersion from the submarine outfall; squares: historical monitoring stations; circles: stations added in this contribution; 1: position of the old Urumea sewer; 2: position of the old Murgita cove outfall; the submarine outfall mouth is between stations 'EMIS' and 'E-S1'); (e) Oslofjord.



2 Materials and methods

2.1. Sites description and anthropogenic pressures

The Bulgarian site is located in the western part of the Black Sea coast and includes the Varna lake, the south navigation canal between the lake and Varna bay (Figure 1a). Varna lake is the largest by volume and deepest lake along the Bulgarian Black Sea coast with a surface area of 17 km^2, maximum water depth of 19 m, and a volume of 166 million m^3. The canal is 6 km long with a maximum depth of 12.5 m. Varna bay is the second largest bay in Bulgaria with a surface area of 20 km^2 and a maximum depth of 18.5 m. Varna is the largest Bulgarian city on the Black Sea; it is a regional centre, accommodating more than 300,000 inhabitants, and an important holiday destination, attracting large numbers of tourists, particularly during the summer season. Urban and industrial infrastructures are well developed along the Varna lake coasts and the lake hosts the largest harbour on the Bulgarian Black Sea. The spatial distribution of urban and industrial infrastructures determines a well defined gradient of decreasing pressures from the inner part of the lake towards the Bay (Table 1).

In Italy (Puglia region, southeastern Italy, Adriatic Sea), the Lesina lagoon is a shallow (maximum water depth 2 m), large (surface area 51 km^2), non-tidal and mesohaline transitional water body (Figure 1b). It is connected with the sea through two narrow and relatively long canals (Acquarotta on the west side and Schiapparo on the east one), and geomorphologically divided into three water basins: a western, a central and an eastern basin. Potentially the lagoon has a low vulnerability to human activities, since its watershed is only 8 times larger than the lagoon surface. However, urban and agricultural wastewater discharges enter the lagoon particularly in the western basin (Table 1), leading to pulse eutrophication events (Vignes *et al.,* 2010). During summer 2008 a strong dystrophic crisis occurred in the western basin, determining hypoxic conditions for a few weeks over an area up to 2.0 km^2, significantly affecting all ecosystem compartments (Specchiulli *et al*., 2009; Vadrucci *et al*., 2009). Nutrient load from wastewaters, reduced hydrodynamism and extreme climate events were advocated as major causes of the dystrophic event (Vignes *et al.,* 2009).

The Mondego estuary is a transitional water body, located on the south eastern Atlantic coast of Europe (Figure 1c). It is a warm-temperate polyhaline system, under the influence of a tidal range varying from 0.35 to 3.3 m, and an annual average water flow of 79 m^3 s^{-1} (27 m^3 s^{-1} in dry years, 140 m^3 s^{-1} in rainy years) (Neto *et al*., 2010). The main channel (north arm) is 21 km long with a surface area of 5.87 km^2, 20% of which is intertidal, and a 2 days' residence time. The south arm is 7 km long with a surface area of 2.71 km^2, 70% of which is intertidal, and a residence time of 5 days (Neto *et al*., 2010). Average depth at high tide varies from 10 m (downstream) to 3 m (upstream) in the north arm, and from 4 m (downstream) to 2 m (upstream) in south arm. In terms of sediment, the small-sized particles (clay and silt) and higher organic matter content dominate at the inner areas of the south arm, with coarse sand sediments being found mostly in the north arm and in the downstream areas of the southern arm (Teixeira *et al*., 2008a). Nutrients constitute the main pressure on the estuary (Marques *et al*., 1993; Neto *et al*., 2008) (Table 1) which, in addition to modifications on the topography of the river bed and the hydrodynamics of the system (Neto *et al*., 2010), stimulated the eutrophication symptoms observed in the past for the south arm (Martins et al. 2001; Marques *et al*., 2003; Neto *et al*., 2008) (Table 1). The establishment of the reconnection between the two channels occurred in 1998 (blocked in 1994), enlarged in 2006 (with reduction of nutrient loading and residence time), and allowed hydrodynamic conditions on both arms to more closely resemble natural conditions. Presently, the main pressures along the Mondego estuary are: (i) high temporal and spatial variability in salinity (natural), and (ii) nutrients and organic matter enrichment (anthropogenic). Specifically on downstream areas (harbours) dredging is also a significant anthropogenic pressure (Table 1).

Within the Basque Country (Bay of Biscay, northern Spain), the Mompás-Pasaia is an exposed, euhaline, shallow (<100 m water depth) coastal water body type (Figure 1d). Since 1976 it has been affected by anthropogenic impacts, especially urban and industrial wastewater discharges (Borja *et al.*, 2006; Tueros *et al.*, 2009) (Table 1). However, in recent times, a water treatment programme has been completed, including three phases (Figure 1d): (i) the elimination of the wastewater discharges from a nearby estuary and their diversion to an outfall discharging at 15 m water depth in Murgita Cove (Figure 1d), between 1996 and 1997; (ii) the diversion, in 2001, of the wastewater discharges from the Urumea sewer, which had discharged directly to the shore since 1976, to the Ulia submarine outfall (discharging at approx. 1 km from the coastline and 50 m water depth); and (iii) the physico-chemical and biological water treatment at a Wastewater Treatment Plant since 2005-2006, previously discharging 5,400 $m^3 h^{-1}$ of wastes through the submarine outfall (Muxika *et al.,* submitted).

The Oslofjord is the largest fjord in the Skagerrak (Norway), going 100 km inland to the city of Oslo (Figure 1e). Approximately 1 million people live in the area. The sediments in Oslo harbour and the innermost part of the fjord are contaminated as a result of industrial activities, boat traffic, urban road traffic, municipal wastewater, small rivers draining from industrial areas and altogether this stressed environment affects the fauna living in both the inner and outer Oslofjord (Rosenberg *et al.* 1987, Josefson *et al.,* 2009) (Table 1). Organic enrichment shows a more variable loading due to distance from urban areas (e.g. Oslo, Drammen, Fredrikstad), river runoff (e.g. Glomma), and local topography with sill and basin areas that influence sedimentation and Total Organic Carbon (TOC) concentrations in sediments.

Table 1. Pressures determined at each location and sampling station (see Figure 1), showing the pressure gradient in the total value and a pressure index, calculated as a mean value of the pressures. Values: 1- low pressure; 2- moderate pressure; 3- high pressure.

Type of pressures	Non-point sources		Pollution			Habitat loss	Industry				Ports		Fisheries		Physico-chemical						
Pressures	**Agricultural diffuse inputs**	[illegible]	[illegible]	[illegible]	[illegible]	**Land-claim**	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	**Fin-Fisheries**	**Shell-fisheries**	**Chlorophyll**	**Nutrients DIN**	**Nutrients P**	**Oxygen**	**Turbidity**	**TOTAL**	**Pressure Index (mean)**
Varna bay																					
V5			3	3	3		3	3	3	3	2									23	2.88
V4			2	3							2									7	2.33
V3			1	2							2	2								7	1.75
V2			1	2							1									4	1.33
V1			1	2							1									4	1.33
Lesina lagoon																					
WSL01	2		2		3								3							10	2.50
WSL02	2		2		2								3							9	2.25
WSL06	2		1		1								3							7	1.75
WSL07	1		1		1								3							6	1.50
WSL03	1												3							4	2.00
WSL04	1												3							4	2.00
WSL05	1												3							4	2.00
Mondego estuary																					
MON-ST23		3		1		3					1		1		2	3	2	2	3	21	2.10
MON-ST18		3		1		3					1		1		1	2	2	2	2	18	1.80
MON-ST9		2		2		1					1				1	2	2	2	3	16	1.78
MON-ST12		1		2		2					1	1	1	2		1	1	1	1	14	1.27
MON-ST2				2		3					1	2	1	2		1	1	1		14	1.56
MON-ST4		1		2		1					1		1	1		1	1	2	1	12	1.20
Basque coast																					
EMIS				2.																2.61	2.61
E-S1				2.																2.16	2.16
WSB01				1.																1.44	1.44
E-S2				1.																1.11	1.11
WSB02				1.																1.15	1.15
E-SW				1.																1.07	1.07
E-NW				1.																1.05	1.05
E-SE				1.																1.02	1.02
E-COL				1.																1.02	1.02
E-NE				1.																1.02	1.02
WSB03				1.																1.01	1.01
E-N				1.																1.00	1.00
WSB04				1.																1.00	1.00
WSB05				1.																1.01	1.01
WSB06				1.																1.00	1.00
WSB07				1.																1.00	1.00
Oslofjord																					
Bn31	2	2	3		3															10	2.50
OF1	2	2	2		2								1							9	1.80
Gl22	2	1	2		1								2							8	1.60
Dk21	2	1	2		2															7	1.75
A36	2	1	1		1								1							6	1.20
YF1	2	1	1		1								1							6	1.20
OF7	1	1	1		1								1							5	1.00
A05	1	1	1																	3	1.00

2.2. Definition of anthropogenic pressure indices

Pressures were quantified (low, medium and high) for each location and sampling station, as partial pressure, total pressure and as a pressure index. The total pressure is the sum of partial pressures, and the pressure index was calculated as a mean value of the pressures.

When available, quantitative data used for defining pressures were obtained from the systems' time-series. Physico-chemical parameters correspond to averaged monthly measurements (surface and/or bottom). Other types of pressures were defined based on professional judgement.

In the Basque coast, quantitative data of the pressure (rate of particles deposited in the sediment from the submarine outfall) exist from a modelisation (Uriarte *et al*., 2004). These quantitative data were rescaled from 1 (the sampled site with the lower number of particles) to 3 (the mean value of the sites with pressure higher than the 99th percentile value, as predicted by the modelisation, i.e. near the submarine outfall) at each station. The quantification of oxygen pressure followed the criteria proposed by Best *et al*. (2007), and was corrected (increasing pressure level) based also on minimum registered values. The freshwater input pressure was defined based on salinity values, following the Venice symposium thresholds, and used in the reverse order of salinity (higher salinity means lower freshwater input pressure). Criteria for nutrients and chlorophyll *a* pressure definitions were based on existent eutrophication classifications (e.g., Bricker *et al*., 1999; Crouzet *et al*., 1999).

2.3. Sampling sites and analyses

Varna bay was sampled on 13th-14th December 2004. Five sampling sites were selected (Figure 1a), specifically taking into account the environmental gradient of nutrients, whose concentrations decrease from the lake to the bay. At each location 5 van Veen grab (0.05 m^2) samples were collected – 4 replicates for benthic analysis and an additional grab for the sediment analysis.

Lesina lagoon has been regularly monitored for the last two years (2008-2009). For this contribution, 7 samples were taken, for sediment and benthic analysis. The stations were selected following the experience achieved with the monitoring programme and the analysis of a dystrophic crisis event in 2008, by setting the stations along a gradient from the western to the eastern basin of the lagoon. Sampling survey of the 7 stations was undertaken between 21st and 23rd September 2009. At each location, four 0.0225 m^2 Ekman grab samples were collected and pooled in order to constitute a sample for the benthic analysis; three replicate samples were collected at each station.

The Mondego estuary has been monitored since 1990 (Teixeira *et al*., 2009; Neto *et al*., 2010). In 2009 the sampling survey of 6 stations was undertaken on the 7th September (Figure 1c), following the natural salinity gradient, and anthropogenically induced nutrient pressure of the

Mondego transitional water system. At each location, six replicates, using a 0.1 m^2 Van Veen grab were collected for benthic analysis, and an additional grab for the sediment analysis.

Mompás-Pasaia has been monitored since 1995 (Muxika *et al.*, submitted), in 9 coastal locations. For this contribution, 7 additional samples were taken, for sediment and benthic analysis (Figure 1d). These new stations were selected after a hydrodynamic analysis was undertaken in a previous study (Uriarte *et al.,* 2004)), which provided the main particle deposition areas and transport direction within the area (see Figure 1d). Hence, this was considered as the main pressure gradient. Sampling of the 16 stations was undertaken on 5th and 20th August 2009. At each location, a 0.04 m^2 Shipek grab was collected for sediment analysis, and three replicates, using a 0.1 m^2 Day grab, were collected for benthic analysis.

The Oslofjord has been sampled for over a century (Petersen, 1915; Rosenberg *et al.,* 1987), and today monitoring is performed under three different programs (Inner Oslofjord, Outer Oslofjord, and long-term monitoring of environmental quality in the coastal regions of Norway). The Oslofjord case study includes 8 stations numbered after their relative distance from Oslo Opera (Figure 1e). Sampling was performed in February 2008 and May-June 2009. Three, four or eight replicate samples were taken with a 0.1 m^2 van Veen grab and sediment samples for TOC were taken with Gemini corer, using the top 0-1 cm for TOC analysis and 0-5 cm for grain size analysis.

All benthic samples were sieved through 1 mm screen and fixed in 4% buffered formalin. Sediment samples were frozen following collection and subsequently most of them processed at the University of Hull. Particle size distribution was determined using a Malvern Mastersizer™ for fractions less than 2 mm and dry sieving through a nest of sieves for coarser particles. The pooled data were processed using GRADISTAT (Blott and Pye, 2001) software to derive statistics such as mean and median grain size, sorting coefficient, skewness, kurtosis and bulk sediment classes (% silt, sand and gravel). Calculations were based on logarithmic Folk and Ward graphical measures. To determine the organic content, dried and pre-weighed samples were placed in a muffle furnace for 4 hours at 480ºC and re-weighed following cooling. Organic content was expressed as percent loss on ignition or TOC.

Animals were sorted and identified to the lowest possible taxonomic level. Specimens were counted, measured (body length) and weighted (dry weight: 60ºC, 72 h; ash-free dry weight: 450ºC, 12 h; except for the Oslofjord samples) and all data were incorporated into a database. Taxonomy was standardized using the European Register of Marine Species (ERMS) (http://www.marbef.org/data/erms.php).

2.4. Metrics and methods calculation

For this contribution, 13 single metrics and 8 of the most common indices used within the Water Framework Directive (WFD) for benthic assessment (see Borja *et al.,* 2009b) were selected. As single metrics abundance, species richness (as number of *taxa*), Shannon's diversity (H'), AMBI (AZTI's Marine Biotic Index; Borja *et al.,* 2000), the 5 Ecological Groups (EG) in AMBI (from sensitive to opportunistic species), Margalef index (d), SN (Rygg, 2006), and Hurlbert indices ES_{100} and ES_{50} (Hurlbert, 1971) were calculated. As multimetric or multivariate methods ISS (Index of Size Spectra; Basset *et al.,* in preparation), BAT (Benthic Assessment Tool; Marques *et al*., 2009; Teixeira *et al.,* 2009), NQI (Norwegian Quality Index, Borja *et al.,* 2007; Josefson *et al.,* 2009), M-AMBI (multivariate AMBI, Borja *et al.,* 2004; Muxika *et al.,* 2007), BQI (Biological Quality Index; Rosenberg *et al.,* 2004), BEQI (Benthic Ecosystem Quality Index; Van Hoey *et al*., 2007a), BITS (Benthic Index based on Taxonomic Sufficiency; Mistri and Munari, 2008), and IQI (Infaunal Quality Index; Prior *et al.,* 2004) were calculated. All of these indices were calculated at the replicate level, except BEQI, which was calculated at sampling station level for methodological reasons. AMBI and M-AMBI were calculated using AMBI software (http://ambi.azti.es). As our objective is only to determine the response of the indices to gradients of pressure, and not to classify them in quality levels, reference conditions for BAT and M-AMBI were defined using the highest values of richness and diversity and the lowest values of AMBI, within the dataset.

In the case of BQI, previous $ES_{50\ 0.05}$ calculation for each species is needed, and the method described in Leonardsson *et al.* (2009) was used. As the amount of data to do this analysis needs to be so high, previous datasets from each of the sampling locations were used. Hence, 144 samples from the Lesina lagoon (www.transitionalwaters.unile.it), 637 samples from Mondego estuary (Teixeira *et al*., 2009; Neto *et al*., 2010), 552 samples from the Basque coast monitoring network (Borja *et al.,* 2009a; Galparsoro *et al.,* 2010), and 2245 coastal samples from Norway (NIVA database) were used for calculating the $ES_{50\ 0.05}$ values. These values, for each location and species, can be consulted as Supplementary Material (Sheet 1).

2.5. Statistical treatment

As the number of replicates was different at each location, mean and standard values were calculated for each sampling station, from the replicates taken. Then, in order to make comparable all the metrics and methods studied, data were standardized, by subtracting the mean value of each location from the sampling station value, and dividing by the standard deviation. Within each of the five locations, sampling stations were ordered in an increasing pressure gradient, according to a preliminary classification based on professional judgement (Table 1). The response of single metrics and assessment methods to the pressure gradient was evaluated using Spearman rank correlation coefficients (ρ). Comparisons between systems were made against the mean value of the pressure (pressure index), and comparisons within each system were made against the total pressure value. Overall, Pearson correlation was used to determine relationships between metrics and methods and between these and environmental variables. A Principal Component Analysis (PCA), using the 13 single metrics, the 8 methods, a pressure index (mean values in Table 1) and environmental variables (Table 2) was performed, after transforming data based upon data exploration (Zuur *et al.,* 2010). This was done by subtracting the mean and dividing by the standard deviation in order to achieve a normal distribution of the variables. All statistical analyses were undertaken using Statgraphics Plus 5.0.

3 Results

The main environmental characteristics of each sampled station can be seen in Table 2. The data show distinct environments and water types, in terms of depth, salinity, grain size, etc., including a lagoon, an estuary, a fjord, and two coastal waters in different ecoregions (Black Sea, Mediterranean, Northeast Atlantic, and North Sea-Skagerrak). Standardized data from all metrics can be consulted in the Supplementary Material (Sheet 2).

Table 2. Environmental characteristics of the sampling sites. Stations are ordered according to the distance from the pressure source, from the closest to the farthest. NA- information not available. TOC- total organic carbon.

Country and water type (name of the site)	Station	Depth (m)	Distance to the pressure (km)	Salinity	Redox potential (mV)	Gravel (%)	Sand (%)	Mud (%)	Organic Content (%)
Bulgarian coast (Varna bay)	V5	-5	2.16	euhaline	NA	0.5	13.5	86.0	6.17
	V4	-5	8.78	euhaline	NA	0.3	45.5	54.2	1.80
	V3	-8	10.77	euhaline	NA	5.3	66.7	28.0	0.86
	V2	-7.5	12.69	euhaline	NA	8	91.2	0.8	0.10
	V1	-17	14.02	euhaline	NA	0.3	12.4	87.3	0.77
Italian lagoon (Lesina)	WSL01	-1	0.60	mesohaline	-429	6.1	55.4	38.5	4.7
	WSL02	-1	0.98	mesohaline	-384	4.3	46.1	49.6	5.6
	WSL03	-1.1	7.76	mesohaline	-382	1.9	58.4	39.8	10.4
	WSL04	-1.1	9.30	mesohaline	-360	4.9	66.7	28.4	9.4
	WSL05	-1.2	11.27	mesohaline	-393	3.6	63.2	33.2	14.0
	WSL06	-1.05	12.79	mesohaline	-384	4.4	70.0	25.5	8.7
	WSL07	-0.6	15.88	mesohaline	-333	0.3	63.5	36.3	9.8
Portuguese estuary (Mondego)	MON-St23	-3	2.72	oligohaline	NA	38.5	59.7	1.7	0.27
	MON-St18	-4	9.10	mesohaline	NA	40.4	59.3	0.3	0.53
	MON-St9	-2	13.04	polyhaline	NA	0.3	48.4	51.3	6.63
	MON-St12	-5	16.17	polyhaline	NA	13.0	84.8	2.2	0.64
	MON-St4	-3	17.21	polyhaline	NA	10.3	89.7	0.0	0.85
	MON-St2	-9	19.53	euhaline	NA	4.2	95.8	0.0	0.35
Basque coast (Mompás-Pasaia)	EMIS	-48	0.17	euhaline	44	0.0	71.8	28.1	1.8
	E-S1	-44	0.17	euhaline	-24	0.0	83.6	16.4	1.6
	WSB01	-50	0.35	euhaline	156	0.0	85.4	14.5	1.5
	E-S2	-36	0.47	euhaline	77	0.2	96.7	3.1	1.0
	WSB02	-53	0.55	euhaline	56	0.5	83.6	15.9	2.4
	E-SW	-38	0.60	euhaline	186	0.1	97.7	2.2	0.9
	E-SE	-41	0.60	euhaline	129	0.0	78.5	21.5	1.5
	E-COL	-34	0.75	euhaline	54	0.0	89.5	10.5	1.1
	E-NW	-58	0.75	euhaline	121	0.9	95.4	3.7	1.3
	E-NE	-54	0.75	euhaline	18	0.9	92.9	6.2	1.5
	WSB03	-56	0.90	euhaline	92	0.1	89.7	10.2	1.5
	E-N	-63	1.10	euhaline	57	0.9	94.1	5.0	1.8
	WSB04	-60	1.60	euhaline	38	0.1	83.4	16.5	1.7
	WSB05	-70	2.75	euhaline	14	0.1	77.1	22.8	2.3
	WSB06	-83	5.00	euhaline	1	0.8	65.2	34.0	3.1

	WSB07	-100	9.00	euhaline	63	0.2	44.8	55.0	5.0
									TOC ($mg\ g^{-1}$)
Norwegian fjord (Oslofjord)	Bn31	-84	7.00	euhaline	NA	NA	NA	56.0	38.5
	Dk21	-100	15.00	euhaline	NA	NA	NA	59.0	28.2
	Gl22	-142	26.00	euhaline	NA	NA	NA	57.0	36.3
	OF7	-212	41.00	euhaline	NA	NA	NA	93.0	23.0
	YF1	-290	73.00	euhaline	NA	NA	NA	94.0	23.1
	OF1	-452	103.00	euhaline	NA	NA	NA	99.0	23.0
	A36	-357	116.00	euhaline	NA	NA	NA	98.0	21.6
	A05	-57	126.00	euhaline	NA	NA	NA	71.0	5.3

It is interesting to note that the pressure index, determined in Table 1, is independent from the environmental variables, when using the whole dataset (Table 3). The unique exception is the significant correlation with the distance, due to the selection of the samples in a spatial pressure gradient. When studying single metrics, the strongest correlations were found between the pressure index and diversity, ES50, SN, ES100, EG I (sensitive species), Margalef and AMBI (Table 3). In all cases, there was a negative correlation between environmental quality and pressure. In the case of methods for the ecological status assessment, the highest correlations were found between pressure and BAT, M-AMBI, NQI, BQI and IQI (correlation ranging from -0.52 to -0.73), with BEQI showing the lowest significant correlation (r: -0.45). In turn, BITS and ISS did not show a significant correlation, at alpha 0.05 (Table 3). Of course, most of the environmental variables, and single and multimetric indices are correlated with each other. The values can be consulted in the complete correlation matrix as Supplementary Material (Sheet 3).

In systems where several pressures were identified (Table 1), almost all single and multimetric indices showed higher Spearman rank correlation coefficients with the overall pressure index than with any specific type of pressure. The comparisons made between systems against the mean value of the pressure (pressure index) can be consulted in the complete correlation matrix supplied as Supplementary Material (Sheet 4).

A detailed analysis of single and multimetric indices' performance by system, allowed seeing that their efficiency was not independent of the type of system. In Varna bay, single and multimetric indices' results were hardly correlated with each other and none of them detected the pressure gradients described. On the contrary, in the Basque coast, the single and multimetric indices were almost all significantly and many of them highly correlated with each other (Supplementary Material, sheets 5 to 9). The multimetric indices significantly correlated with the pressure gradients also varied according to the system (Table 4).

Table 3. Pearson correlation (first number), number of pairs of data (second number) and p-value (third number), between pressure index (as mean value in Table 1) and distance to the main source of pressure and environmental variables (values in Table 2), single metrics and multimetric or multivariate methods. Bold and underlined values show significant correlation for p<0.001, and underlined values for p<0.05. EG: Ecological Group.

Environmental	Pressure	Distance	Single	Pressure	Distance	Single	Pressure	Distance	Method	Pressure	Distance
Depth	-0.05	0.09	**AMBI**	0.49	-0.23	**EGIV**	-0.25	0.23	**BAT**	-0.73	0.38
	42	42		42	42		42	42		42	42
	0.730	0.562		**0.001**	0.139		0.116	0.137		**0.000**	0.014
Distance	-0.41		**Margalef**	-0.54	0.32	**EGV**	0.07	0.00	**BEQI**	-0.45	0.29
	42			42	42		42	42		35	35
	0.007			**0.000**	0.037		0.676	0.977		0.006	0.088
Mud	0.13	0.41	**Diversity**	-0.75	0.39	**ES100**	-0.64	0.35	**BITS**	-0.06	-0.09
	42	42		42	42		42	42		42	42
	0.407	0.006		**0.000**	0.010		**0.000**	0.023		0.691	0.585
Organic Matter	0.12	0.29	**EGI**	-0.59	0.29	**ES50**	-0.69	0.37	**BQI**	-0.54	0.34
	42	42		42	42		42	42		37	37
	0.435	0.060		**0.000**	0.061		**0.000**	0.017		**0.001**	0.039
Redox potential	-0.08	-0.59	**EGII**	-0.36	0.12	**Abundance**	0.49	-0.29	**IQI**	-0.52	0.23
	23	23		42	42		42	42		40	40
	0.732	0.003		0.019	0.436		**0.001**	0.060		**0.001**	0.157
Sand	-0.12	-0.64	**EGIII**	0.25	-0.13	**Richness**	-0.37	0.27	**ISS**	-0.36	0.47
	42	42		42	42		42	42		18	18
	0.450	**0.000**		0.113	0.429		0.015	0.084		0.141	0.049
						SN	-0.68	0.34	**MAMBI**	-0.72	0.37
							42	42		42	42
							0.000	0.027		**0.000**	0.015
									NQI	-0.65	0.32
										42	42
										0.000	0.041

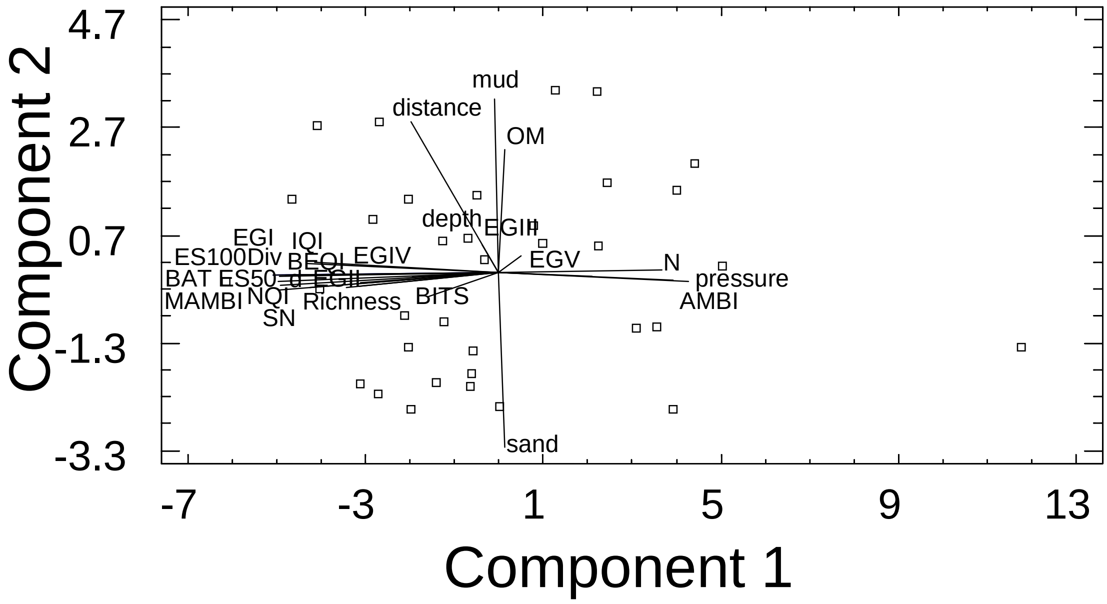
In some of the systems where several types of pressures were identified, Lesina lagoon, Mondego estuary and Oslofjord, it was also observed that the single and multimetric indices tested had different sensitivities towards specific types of pressure (Table 4). Usually, for the multimetric indices that were significantly correlated with the pressure gradient it was observed that they were more integrative than the single metrics, since they could often detect the overall pressure index and also several of the specific types of pressures identified, while single metrics presented higher variability in their capability of detecting multiple pressures or the overall pressure index.

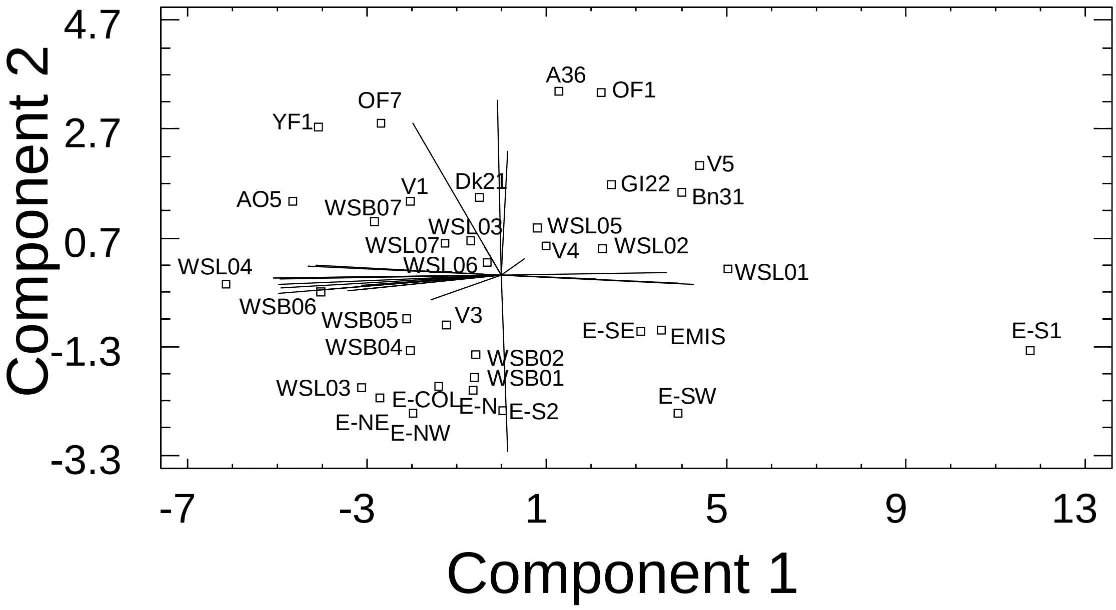
Table 4. Spearman rank correlation coefficient within each system: correlation of single metrics and multimetric methods with pressure (Total pressures and specific type of pressures: A – Non-point sources; B – pollution; C – Habitat loss; D – Industry; E – Ports; F – Fisheries; G – Physico-chemical); correlations between total pressure and pressure index (Pi, mean), and between type of pressure and total pressure. Spearman rank correlation coefficient (first number), and p-value (third number). Bold values show significant correlation for p<0.05.

System	Pressures	Single metrics													Multimetrics								Pressures	
		% EG I	% EG II	% EG III	% EG IV	% EG V	AMBI	Richness	Diversity	d	N	SN	ES100	ES50	ISS	BAT	NQI	M-AMBI	BITS	BQI	BEQI	IQI	Total	Pi
Varna Bay (n=5; except BEQI n=4)	Total	-0.95	-0.95	0.95	0.26	-0.32	0.63	-0.53	-0.53	-0.53	-0.53	-0.53	-0.53	-0.53	-0.21	-0.95	-0.95	-0.95	0.00		-0.32	-0.95		0.97
		0.058	0.058	0.058	0.598	0.527	0.206	0.292	0.292	0.292	0.292	0.292	0.292	0.292	0.673	0.058	0.058	0.058	1.000		0.584	0.058		0.052
	B	-0.89	-0.89	0.89	-0.11	-0.67	0.22	-0.89	-0.89	-0.89	-0.67	-0.89	-0.89	-0.89	-0.45	-0.89	-0.67	-0.89	0.22		-0.63	-0.67	0.83	
		0.074	0.074	0.074	0.823	0.180	0.655	0.074	0.074	0.074	0.180	0.074	0.074	0.074	0.371	0.074	0.180	0.074	0.655		0.273	0.180	0.099	
	D	-0.71	-0.71	0.71	-0.35	-0.71	0.00	-0.71	-0.71	-0.71	-0.71	-0.71	-0.71	-0.71	0.00	-0.71	-0.71	-0.71	0.00		-0.77	-0.71	0.75	
		0.157	0.157	0.157	0.480	0.157	1.000	0.157	0.157	0.157	0.157	0.157	0.157	0.157	1.000	0.157	0.157	0.157	1.000		0.180	0.157	0.136	
	E	-0.87	-0.87	0.87	0.31	-0.21	0.67	-0.36	-0.36	-0.36	-0.46	-0.36	-0.36	-0.36	-0.05	-0.87	-0.97	-0.87	-0.10		-0.20	-0.97	0.97	
		0.081	0.081	0.081	0.538	0.682	0.182	0.473	0.473	0.473	0.356	0.473	0.473	0.473	0.918	0.081	0.051	0.081	0.837		0.729	0.051	0.052	
Oslofjord (n=8)	Total	-0.69	**-0.90**	0.31	0.44	0.43	**0.90**	0.42	**-0.89**	-0.30	**0.87**	**-0.78**	**-0.83**	**-0.85**		**-0.81**	**-0.91**	**-0.75**	0.07	-0.50	-0.23	**-0.83**		**0.97**
		0.066	**0.018**	0.410	0.241	0.260	**0.018**	0.267	**0.019**	0.428	**0.021**	**0.039**	**0.029**	**0.025**		**0.031**	**0.016**	**0.046**	0.849	0.183	0.534	**0.029**		**0.010**
	A	-0.69	**-0.77**	0.46	0.31	0.44	**0.77**	0.31	-0.69	-0.31	**0.77**	**-0.77**	**-0.77**	-0.69		-0.69	**-0.85**	-0.62	-0.15	-0.39	-0.16	**-0.77**	**0.93**	
		0.066	**0.041**	0.221	0.414	0.242	**0.041**	0.414	0.066	0.414	**0.041**	**0.041**	**0.041**	0.066		0.066	**0.025**	0.103	0.683	0.307	0.681	**0.041**	**0.014**	
	B	-0.52	**-0.85**	0.43	0.33	0.21	**0.77**	0.36	**-0.90**	-0.36	**0.74**	-0.71	-0.72	-0.74		-0.72	**-0.76**	-0.68	0.29	-0.45	-0.21	-0.68	**0.94**	
		0.173	**0.025**	0.256	0.381	0.577	**0.041**	0.346	**0.018**	0.346	**0.051**	0.060	0.055	0.051		0.055	**0.044**	0.074	0.436	0.230	0.579	0.074	**0.013**	
	F	-0.30	0.07	-0.46	0.00	0.66	0.18	0.18	-0.03	0.05	0.27	-0.08	-0.33	-0.37		-0.21	-0.12	-0.12	-0.53	-0.14	-0.23	-0.12	0.05	
		0.427	0.863	0.227	1.000	0.082	0.629	0.629	0.945	0.890	0.469	0.836	0.388	0.334		0.581	0.756	0.756	0.157	0.704	0.544	0.756	0.890	
Lesina lagoon (n=7)	Total	-0.63	-0.42	0.67	-0.22	-0.43	0.74	-0.70	-0.37	**-0.82**	0.52	**-0.82**	**-0.82**	-0.59	-0.59	-0.56	**-0.96**	-0.56	0.30	-0.30	**-0.93**	-0.15		0.42
		0.123	0.299	0.102	0.586	0.293	0.069	0.085	0.364	**0.046**	0.204	**0.046**	**0.046**	0.146	0.146	0.173	**0.018**	0.173	0.468	0.468	**0.023**	0.717		0.300
	A	-0.29	-0.35	0.43	-0.14	-0.16	0.43	**-0.87**	-0.29	**-0.87**	0.29	**-0.87**	**-0.87**	-0.58	-0.58	-0.43	**-0.87**	-0.43	0.43	-0.29	**-0.87**	-0.14	**0.90**	
		0.480	0.387	0.289	0.724	0.697	0.289	**0.034**	0.480	**0.034**	0.480	**0.034**	**0.034**	0.157	0.157	0.289	**0.034**	0.289	0.289	0.480	**0.034**	0.724	**0.028**	
	B	-0.71	-0.43	0.73	-0.26	-0.51	**0.80**	-0.62	-0.39	-0.77	0.58	-0.77	-0.77	-0.58	-0.58	-0.58	**-0.95**	-0.58	0.26	-0.26	**-0.90**	-0.11	**0.99**	
		0.082	0.295	0.074	0.521	0.215	**0.049**	0.130	0.336	0.060	0.155	0.060	0.060	0.155	0.155	0.155	**0.019**	0.155	0.521	0.521	**0.028**	0.783	**0.015**	
Mondego estuary (n=6; except BEQI n=4)	Total	-0.72	-0.64	0.84	-0.74	-0.55	0.64	**-0.93**	**-0.90**	**-0.99**	0.35	**-0.99**	**-0.90**	**-0.90**	-0.23	**-0.90**	-0.84	**-0.90**	0.46	**-0.96**		0.32		**0.99**
		0.105	0.154	0.060	0.098	0.218	0.154	**0.038**	**0.045**	**0.028**	0.437	**0.028**	**0.045**	**0.045**	0.604	**0.045**	0.060	**0.045**	0.300	**0.033**		0.584		**0.028**
	A	**-0.88**	-0.79	**0.97**	-0.66	-0.71	0.79	-0.79	**-0.97**	**-0.88**	0.53	**-0.88**	**-0.97**	**-0.97**	-0.44	**-0.97**	**-0.88**	**-0.97**	0.53	**-0.94**		0.95	0.85	
		0.048	0.076	**0.030**	0.142	0.114	0.076	0.076	**0.030**	**0.048**	0.236	**0.048**	**0.030**	**0.030**	0.324	**0.030**	**0.048**	**0.030**	0.236	**0.036**		0.100	0.057	
	B	0.62	0.41	-0.83	0.66	0.62	-0.41	0.62	0.83	0.83	-0.41	0.83	0.83	0.83	0.41	0.83	0.62	0.83	-0.41	0.84		0.00	-0.84	
		0.165	0.355	0.064	0.140	0.165	0.355	0.165	0.064	0.064	0.355	0.064	0.064	0.064	0.355	0.064	0.165	0.064	0.355	0.060		2.000	0.060	
	C	-0.06	0.25	0.28	-0.49	-0.25	-0.25	-0.28	-0.28	-0.49	-0.12	-0.49	-0.28	-0.28	-0.12	-0.28	-0.06	-0.28	0.12	-0.39		-0.95	0.56	
		0.890	0.581	0.535	0.271	0.581	0.581	0.535	0.535	0.270	0.783	0.270	0.535	0.535	0.783	0.535	0.890	0.535	0.783	0.382		0.100	0.208	
	E	0.68	0.85	-0.68	0.11	0.34	-0.85	0.44	0.68	0.44	-0.78	0.44	0.68	0.68	0.17	0.68	0.68	0.68	-0.17	0.57		-0.95	-0.41	
		0.131	0.059	0.131	0.810	0.450	0.059	0.326	0.131	0.326	0.082	0.326	0.131	0.131	0.706	0.131	0.131	0.131	0.706	0.206		0.100	0.357	
	F	0.79	**0.97**	-0.71	0.19	0.26	**-0.97**	0.71	0.71	0.62	-0.62	0.62	0.71	0.71	-0.09	0.71	0.79	0.71	-0.09	0.67		-0.95	-0.63	
		0.076	**0.030**	0.114	0.675	0.554	**0.030**	0.114	0.114	0.167	0.167	0.167	0.114	0.114	0.844	0.114	0.076	0.114	0.844	0.133		0.100	0.161	
	G	-0.71	**-0.94**	0.77	-0.39	-0.37	**0.94**	-0.83	**-0.89**	-0.77	0.77	-0.77	**-0.89**	**-0.89**	-0.14	**-0.89**	**-0.94**	**-0.89**	0.37	-0.84		**1.00**	0.75	
		0.110	**0.035**	0.085	0.378	0.406	**0.035**	0.064	**0.048**	0.085	0.085	0.085	**0.048**	**0.048**	0.749	**0.048**	**0.035**	**0.048**	0.406	0.060		**0.000**	0.092	
Basque coast (n=16)	Total	**-0.68**	-0.01	0.28	**-0.79**	**0.77**	0.22	**-0.76**	**-0.66**	**-0.72**	0.17	**-0.66**	**-0.72**	**-0.66**		**-0.63**	-0.49	**-0.64**	-0.08	**-0.83**	**-0.63**	**-0.57**		**1.00**
		0.009	0.977	0.271	**0.002**	**0.003**	0.387	**0.003**	**0.011**	**0.005**	0.523	**0.011**	**0.005**	**0.010**		**0.015**	0.058	**0.014**	0.765	**0.001**	**0.015**	**0.026**		**0.000**
	B	**-0.68**	-0.01	0.28	**-0.79**	**0.77**	0.22	**-0.76**	**-0.66**	**-0.72**	0.17	**-0.66**	**-0.72**	**-0.66**		**-0.63**	-0.49	**-0.64**	-0.08	**-0.83**	**-0.63**	**-0.57**	**1.00**	
		0.009	0.977	0.271	**0.002**	**0.003**	0.387	**0.003**	**0.011**	**0.005**	0.523	**0.011**	**0.005**	**0.010**		**0.015**	0.058	**0.014**	0.765	**0.001**	**0.015**	**0.026**	**0.000**	

From the PCA, component 1, which explains 47.7% of the total variability, is related to the pressure gradient (Figure 2a) taking into account all locations except the Mondego estuary, due to the absence of some variables (e.g. BEQI, IQI). In turn, component 2, which explains 12.5% of the total variability, is related with some environmental variables, such as the percentage of mud or organic matter (Figure 2a). Hence, pressure index, abundance and AMBI increase with increasing component 1 values, and most of the multimetric and single methods are on the opposite part of the axis. This results in a clear gradient of stations distribution, with those close to the main pressure source on the positive values of the first axis, and those less affected on the negative values of the axis (Figure 2b).

Figure 2. Principal Component Analysis, using the pressure index, environmental variables, single and multimetric methods (a), together with the position of sampling stations (b) (see Figure 1). OM- Organic Matter; EG- Ecological Group.





The regression models, including all available data from the locations, for each metric or multimetric method, show that those detecting the pressure gradient with higher correlations are: Shannon's diversity, ES50, SN and the Ecological Group I, as single metrics (Figure 3), and BAT, M-AMBI and NQI, as multimetric or multivariate methods (Figure 4). This result indicates that these metrics are showing clear gradients of degradation within each system.

Figure 3. Regression between the pressure index (see Table 1) and some selected single metrics, in assessing the benthic status.

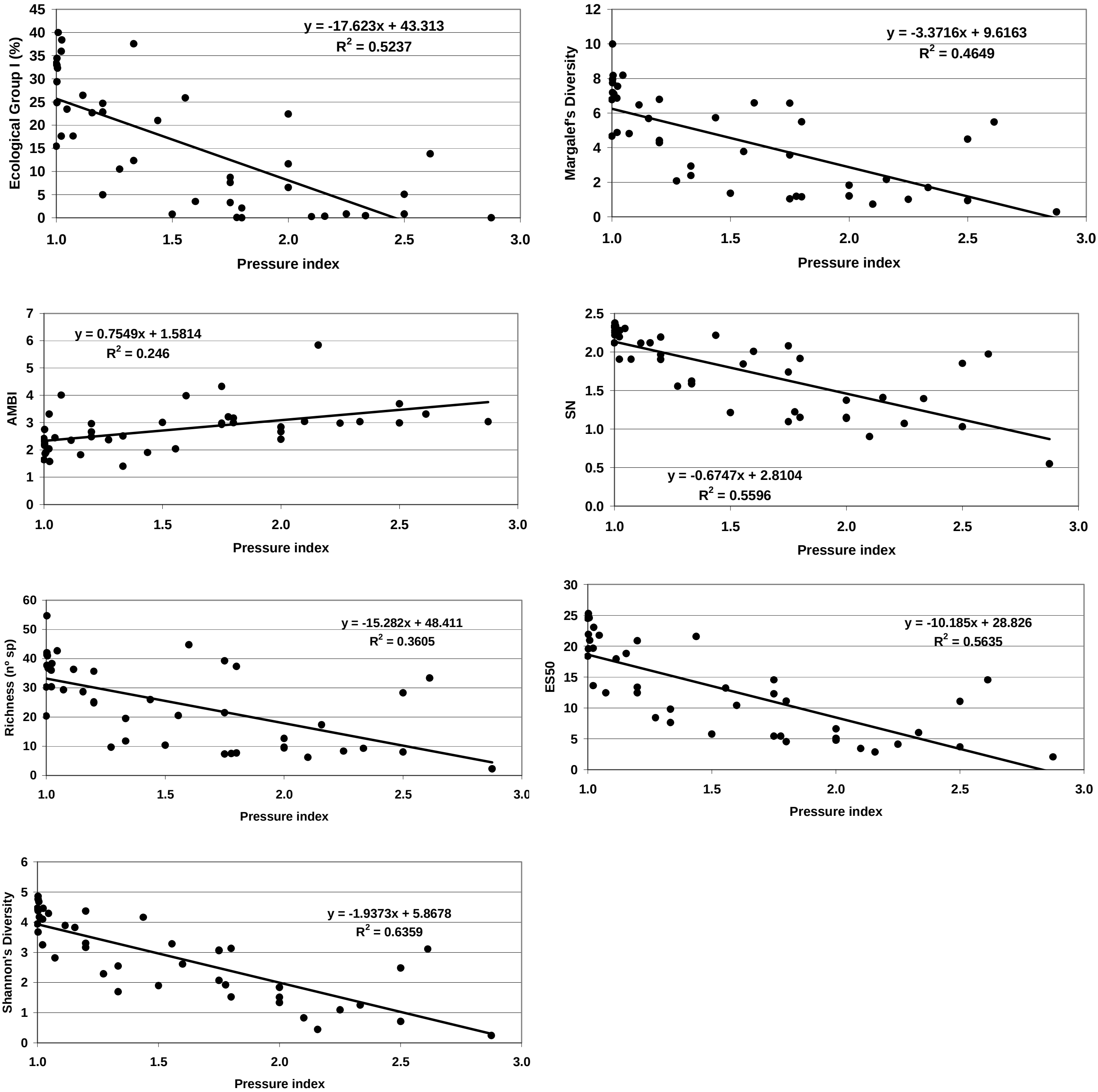
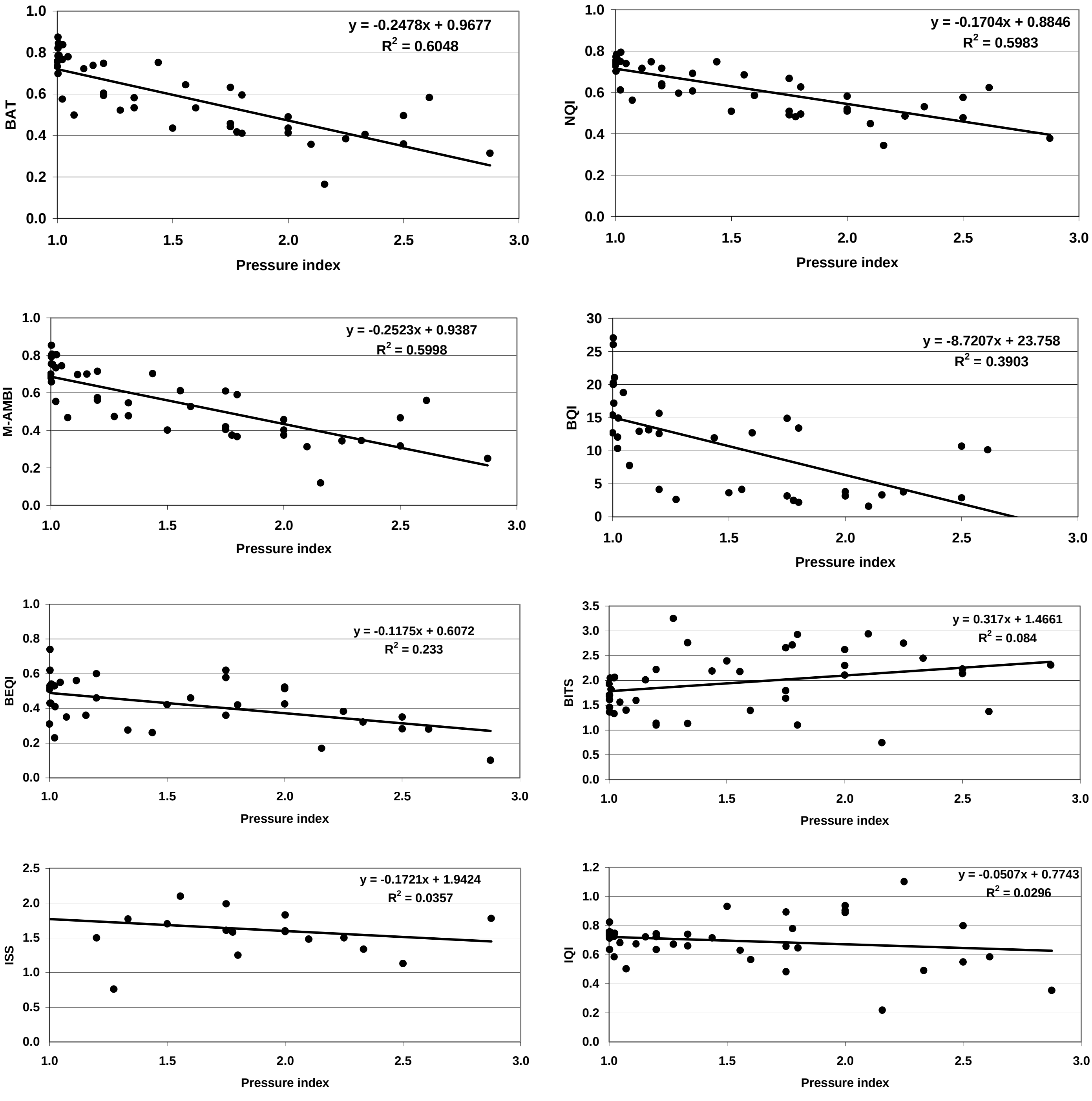
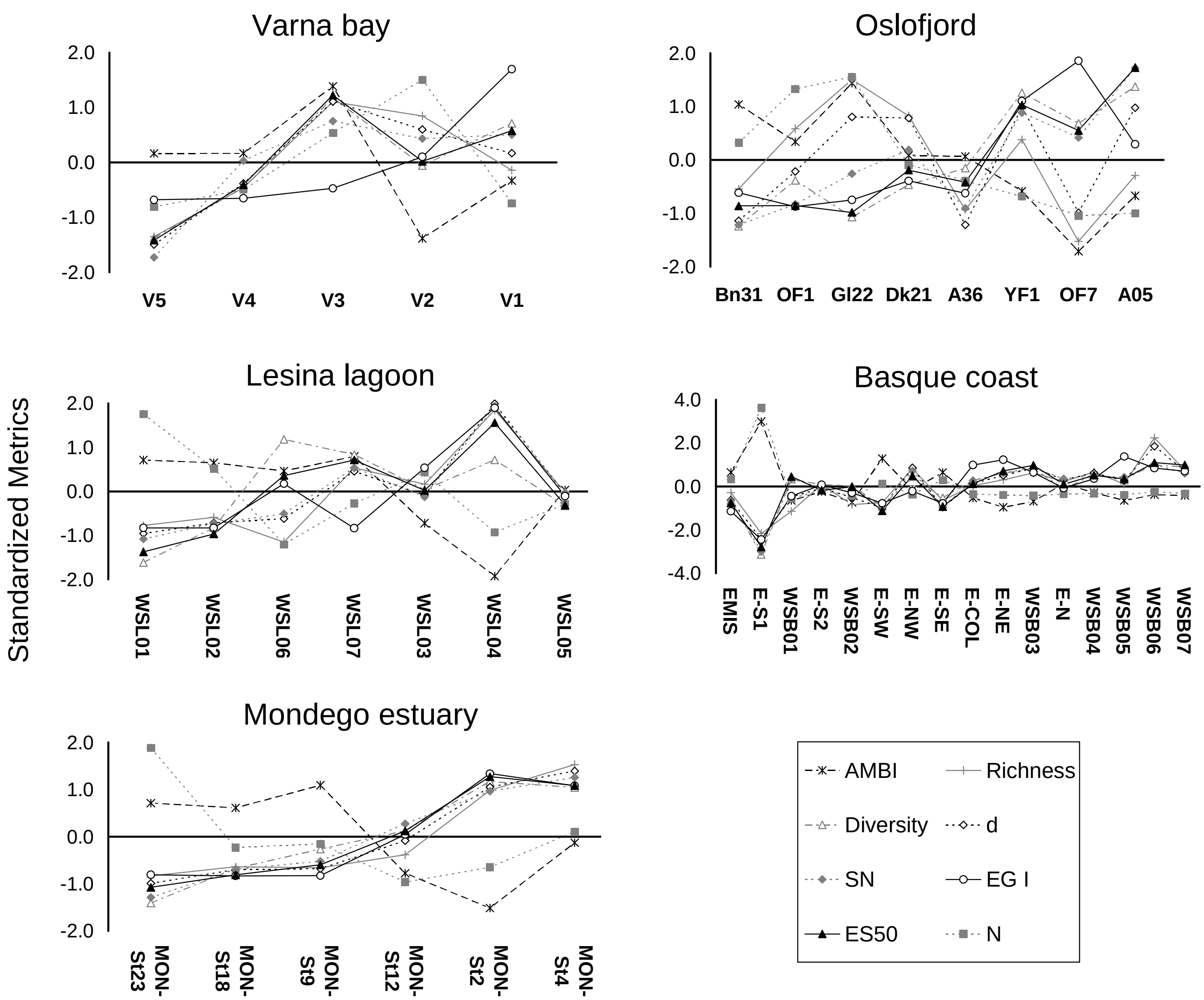


Figure 4. Regression between the pressure index (see Table 1) and multimetric or multivariate methods, in assessing the benthic status.



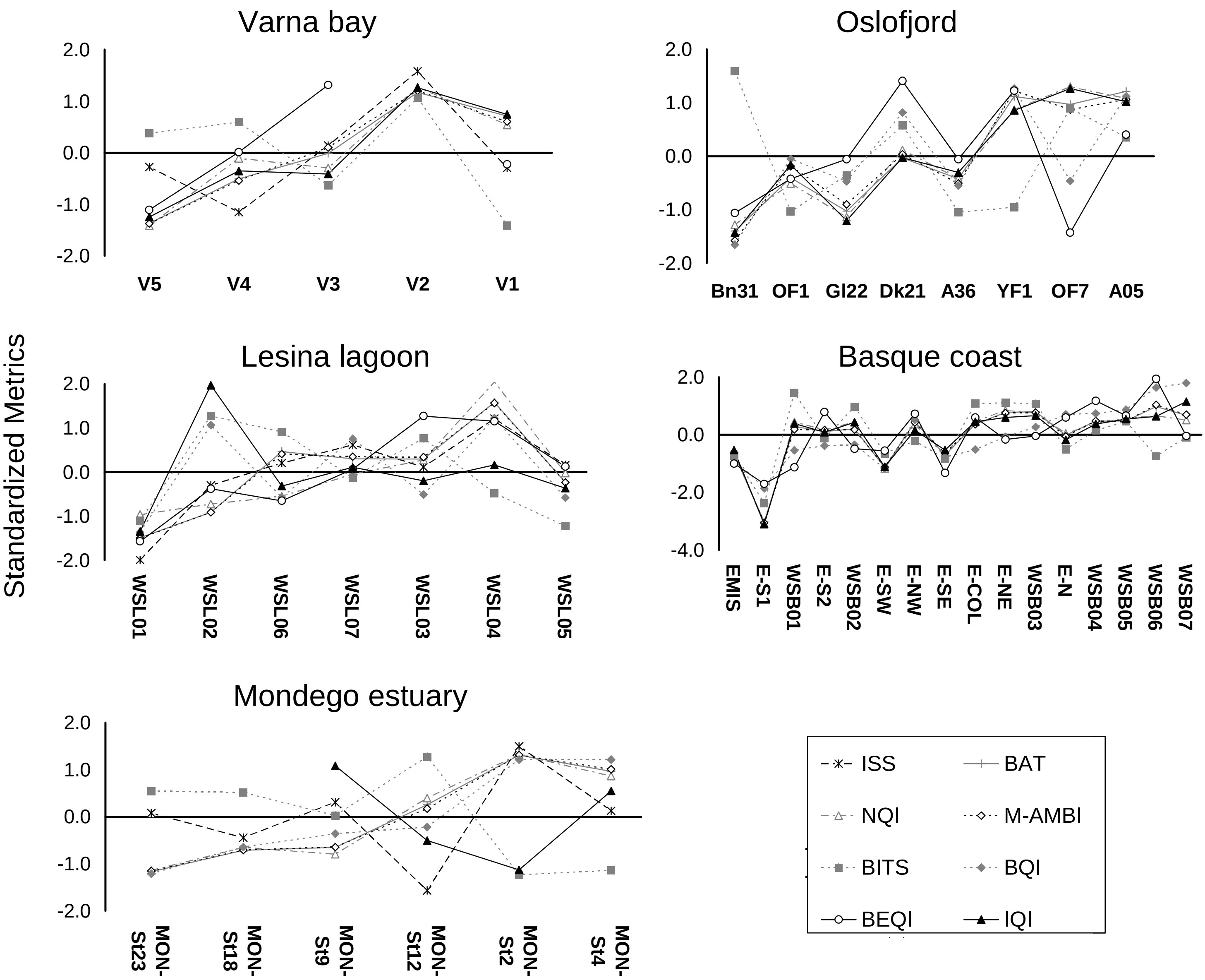
When single metrics are represented following the pressure gradient shown in Table 1, two patterns can be seen (Figure 5): (i) AMBI (this metric has decreasing values with increasing quality status) and N tend to decrease with the decreasing pressure gradient, except in the case of N in Varna bay; and (ii) in turn, the rest of the single metrics tend to increase with the decreasing pressure gradient, being very consistent the pattern in all locations. Only station A36, outside Oslofjord, shows lower values, of single metrics, than expected in the pressure gradient (Figure 5).

Figure 5. Standardized single metrics, following the pressure gradient determined in Table 1, at each location. d- Margalef index; EG I- Ecological group I (sensitive species); N- abundance.



Regarding multimetric and multivariate methods, several patterns can be detected (Figure 6): (i) BITS decreases with the decreasing pressure gradient, when the contrary would be expected. This pattern is clear in Varna bay, Lesina and Mondego, and, at some extent, in Oslofjord; (ii) ISS shows the expected pattern (increasing with decreasing pressure) in Varna and Lesina, but not in Mondego; (iii) NQI, BAT, M-AMBI and BQI show the expected pattern of increasing values with decreasing pressure in all cases; (iv) BEQI shows the same pattern, except in Varna (probably linked to the selection of the reference station); and (v) IQI shows the expected pattern in all locations except in Mondego and Lesina, which are both transitional waters.

Figure 6. Standardized multimetric methods, following the pressure gradient determined in Table 1, at each location.



4 Discussion

Single metrics tested in this investigation can be grouped depending on their ecological basis: (i) some of the metrics are based upon the taxonomical response to human disturbance (e.g. AMBI, ES_{50}, ES_{100}, the five EG); (ii) some are related to the concept of diversity (Shannon or Margalef); (iii) some with the number of individuals (abundance and SN) and (iv) the number of taxa (richness).

In turn, the multimetric or multivariate methods tested can be grouped in three types of benthic indices with fundamental differences in the way they were derived: (i) those based upon species sensitivity to disturbance, either incorporating AMBI or $ES_{50\ 0.05}$, together with some measure of diversity, species richness and abundance, and compared to reference conditions (e.g. BQI, IQI, M-AMBI, BITS, BAT, NQI); (ii) that based on abundance, species richness, biomass and similarity to pre-determined reference conditions (BEQI); and (iii) indices based on the size spectra (or individual size distributions, White *et al.,* 2007) such as ISS, which represents a non-taxonomically based metric. These indices are derived from the distribution of individuals into size classes directly, as simple metrics (ISD, Reizopoulou and Nicolaidou, 2004, 2007), or including a measure of sensitivity of macroinvertebrate size classes to stress (ISS, Basset *et al.,* in preparation).

Hence, the responses of these methods to the pressure gradient, as shown in Figure 4, can be related primarily with the way in which they are derived. The first group includes those indices (M-AMBI, BAT, BQI, etc.), which are close related in its design, mainly based upon sensitivity to organic enrichment and oxygen depletion (following the model of Pearson and Rosenberg, 1978). However, they have demonstrated also its response to other forms of disturbance, such as dredging, pollutants, etc. (Muxika *et al.,* 2005; Borja *et al.,* 2009a; Josefson *et al.,* 2009; Leonardsson *et al.,* 2009 ; Pinto *et al.,* 2009 ; Teixeira *et al.,* 2009; Neto *et al.,* 2010). Another method included in this group, BITS, which was developed primarily for non-tidal lagoons (Mistri and Munari, 2008), does not respond to the pressure gradient, even in the Lesina lagoon. Although it has been successfully tested in other lagoons, detecting oxygen and nutrients gradients (Munari *et al.,* 2009), correlating significantly with M-AMBI (Munari and Mistri, 2010), in this study they do not correlate (r: 0.163, n: 42 cases, p: 0.3, see Supplementary Material). Differences in the strength of pressures, which were relatively weak in our Lesina study case, may account for the differences observed in the behaviour of BITS.

The second group (BEQI alone) is based upon the comparison of sampling data with reference conditions for each ecotope (van Hoey *et al.,* 2007). Hence, it needs sufficient data to explicitly account for spatial and temporal variation in community structure. This level of information was not available for two sites considered in this study (Varna bay and the Mondego estuary) and area-specific information would be required to accurately adapt an index which had been developed based on the ecology of a specific region. Critically, the accurate calculation of BEQI is highly dependent on sampling design and it is essential that the ecotopes within the study area, and their equivalent reference stations, are identified before sampling is carried out. Therefore, the index calculation may have been subject to a significant degree of error.

The third group (ISS alone) is based upon empiric evidences that large-size species are more sensitive than small ones to anthropogenic stress stem from the early work of Person and Rosenberg (1978), being common in the literature on marine (Warwick, 1984), freshwater (Strayer, 1986; Solimini *et al*., 2001) and transitional water (Basset *et al*., 2004) ecosystems. The rationale of these relationships is that, being stress defined as any source of negative influence on the energy flow at the individual level (i.e. scope for growth, Calow, 1989), it is likely to affect size spectra through the cause-effect relationship between individual body size and individual energetics (e.g., Peters, 1983). Different components of size spectra of benthic macroinvertebrate in transitional waters, such as shape (skewness, Reizopoulou and Nicolaidou, 2004, 2007), body size diversity (Gascón *et al*., 2009), size distribution width (Reizopoulou and Nicolaidou 1996; Basset *et al.,* 2004), central tendency and upper boundary (Basset *et al*., 2008), have already been observed to respond to nutrient and organic enrichment (but see also Schwinghamer, 1988; for partially contrasting evidences) and chemical pollution.

Indices of size spectra have a number of advantages over taxonomically based indices. Body size is relatively easy to assess, it does not require specialist expertise in taxonomy and it is easy to intercalibrate among laboratories. On the other hand, indices of size spectra have also a number of disadvantages. Assessment of body mass on large samples is a time-consuming process, as far as more complete and accurate lists of weight per length relationships are available; moreover, the occurrence of large species at low densities is likely to introduce bias in mono-metric descriptions of size spectra, which are also likely to be more sensitive to small samples than taxonomically based indices. The ISS sensitivity to sample size was likely to be a main source of variability of the ISS assessment of the Mondego ecological status; in fact, the absence of ISS fit in the Mondego was mainly related to two stations, stations 2 and 12, where a very low number of individuals was globally measured (23 and 17 respectively). Under these conditions, random occurrence of few large individuals may affect substantially the ecological status assessment with ISS. In fact, when these two stations are removed from the analysis, ISS shows a significant correlation with the pressure index (r: -0.57; p-value: 0.021), which is in the same range that other assessment methods, such as BEQI, BQI or NQI (see Table 3). Hence, probably this fact prevents the calculation of ISS with abundances lower than 25 individuals. This precautionary recommendation is similar to others when calculating some benthic indices e.g. in the case of AMBI (Borja and Muxika, 2005).

In general, the response of most of the indicators to pressure was consistent with index scores increasing in relation to decreasing pressure. This was largely the case for all sites (although there were exceptions) for the univariate metrics (except N and AMBI, which ordinarily increase scores with increasing pressure), NQI, BAT, and M-AMBI. Additionally, these metrics also responded in a similar manner when applied to the whole data set. This result is encouraging and, together with the poor correlation between indicator score and environmental parameters, indicates that these indices are, to some extent, independent of habitat type and geographic region. Nevertheless, the low correlations observed between indices, both single and multimetrics, within particular systems (Supplementary Material, sheets 5 to 9) indicate that some calibration is required before assessing new habitats. When assessing the suitability of a range of benthic indices in the evaluation of aquaculture impacts, across Europe, a similar result was found for some of them (abundance, richness, diversity, AMBI, etc.) (Borja *et al.,* 2009d). In turn, Grémare *et al.* (2009) found contradictory results applying AMBI and BQI to a huge dataset including the whole Europe (although in this particular case they did not test pressure gradients).

Possible explanations for differences in indicator response include: (i) the reference conditions associated to each method; and (ii) the number of pressures within each system, which can mask the main pressure gradient.

Reference conditions are one of the major issues when assessing the ecological status (Muxika *et al.,* 2007). In this investigation, in which there are different aquatic systems at European scale, it is particularly difficult to find continental reference conditions as demonstrated for riverine benthic communities (Herlihy *et al.,* 2008). Despite of this, reference conditions determined for BAT, M-AMBI and NQI have been adequate to catch the pressure gradient, including all systems. In turn, exceptions to the above pattern include the low performance of IQI and BITS in the Mondego estuary and the Lesina lagoon, with some inconsistencies in Varna bay and Oslofjord. BEQI also did not perform as expected in Varna bay and ISS did perform as expected in the Mondego estuary. Some of these inconsistencies can be explained by the difficulty of setting reference conditions in transitional waters (Teixeira *et al.,* 2008b), due to the Estuarine Quality Paradox (Dauvin, 2007; Elliott and Quintino, 2007), which makes difficult to differentiate between natural and anthropogenic stress (Neto *et al.,* 2010). Many of the characteristics (e.g. low salinity, fine grained, organic rich sediments, periods of low oxygen, low species diversity and small organisms) of these transitional environments resemble disturbed conditions in coastal areas. Mistri and Munari (2008) highlight the ways in which these characteristics can lead to erroneous evaluations. Dauvin (2007) stated that most indices are based on the abundance of stress tolerant species yet those stressors occur naturally in transitional waters which support high densities of stress tolerant species. However, some of the single and multimetric methods used here are able to detect the human pressure gradients, both in coastal and transitional waters.

Regarding the number of pressures, a meta-analysis of the interactive and cumulative effects of multiple human stressors in marine systems, undertaken across 171 studies, revealed a significant overall synergistic interaction effect (Crain *et al.,* 2008). This indicates that cumulative effects of multiple stressors will often be worse than expected based on single stressor impacts. Hence, in our study Mondego (12 different pressures) and Varna bay (9 pressures) would be more susceptible to interactions and cumulative effects. In turn, the Basque coast, with a single pressure, would present a clearer pressure-impact response, occupying Oslofjord (5 pressures) and Lesina lagoon (4 pressures) an intermediate situation. On the other hand, since patterns of metrics variation with total pressure and average pressure estimates were very consistent, the achieved results do not seem to be affected by the heterogeneity among study cases in type and number of recorded pressures. The impact of multiple stressors on marine systems will depend not only on species-level responses, but additionally on species interactions, species diversity and redundancy, trophic complexity, ecological history, and ecosystem type (Crain *et al.*, 2008). Hence, complex ecosystems, such as estuaries and lagoons, can show also more complex responses in some indicators. Therefore, and as suggested by our results (Table 4), the application of multimetric methods (WFD requirement) increases the probability of a correct evaluation of the ecological conditions of the system.

5 Conclusions

From the analyses undertaken, some encouraging results have been found, demonstrating that the different indices are largely consistent in their response to a pressure gradient, except in some particular cases (i.e. BITS, in all cases, or ISS when a low number of individuals is present). Inconsistencies between indicator responses were mostly in transitional waters (i.e. IQI, BEQI), highlighting the difficulties of the generic application of indicators to both transitional (estuaries, lagoons) and marine (coasts, fjords) environments. However, some of the single (i.e. ecological groups approach, diversity, richness, SN) and multimetric methods (i.e. BAT, M-AMBI, NQI, and ISS, the latter accounting for the sample size cited restrictions) were able to detect such gradients both in transitional and coastal environments. Finally, this study highlights the importance of survey design and good reference conditions for some indicators and systems (i.e. estuaries and lagoons), which should be addressed in further investigations. In this context, the correct identification and quantification of pressures acting on a system are crucial to: (i) indices' calibration; and (ii) the establishment of successful monitoring and management actions. Not all indicators can be successfully applied in hindsight (e.g. BEQI) or to all data sets.

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