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1 **The robustness of ecological indicators to detect long-term changes in**
2 **the macrobenthos of estuarine systems.**

3
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11
12
13
14 **Abstract**

15 Accurate and reliable benthic quality indicators are in great demand following the
16 recent developments and the strict time schedule for implementing the European Water
17 Framework Directive. The Mondego estuary has experienced a progressive deterioration
18 during the 1990's, followed by a partial ecological recovery due to restoration measures in
19 1997/1998. We have used the estuary as a model system to test the performance and
20 robustness of a set of ecological indicators in highlighting the changes in the ecological
21 state of intertidal areas. Over a period of 17 years (1985-2002), we calculated Margalef,
22 Shannon-Wiener, Berger-Parker, Taxonomic Distinctness measures, AZTI's Marine Biotic
23 Index, Infaunal Trophic Index, and Eco-Exergy based indices and tested differences
24 across periods characterised by different anthropogenic disturbance. We combined
25 temporal data within three periods: before, during and after disturbance, based on

26 progressive information on the changes in the extended type of anthropogenic
27 disturbance. Indices were then compared with biological and abiotic descriptors
28 (macroalgae, macrophytes, benthic macrofauna, nutrients concentration, sediment grain
29 size and total organic carbon). We found great disparity in the indicators ability to capture
30 temporal changes, showing distinct performances at each site. At the *Z. noltii* site, only
31 Margalef, Total Taxonomic Distinctness and the thermodynamically based indices
32 captured temporal changes, despite giving higher values during the disturbance period. At
33 the bare sediment site, Taxonomic Distinctness, ITI, Shannon-Wiener, Berger-Parker,
34 AMBI and the TBI were able to distinguish between periods, in agreement with the
35 differences observed analysing the macrobenthic assemblages. Furthermore, Taxonomic
36 Distinctness was not robust enough to detect any temporal or spatial change. We thus
37 suggest further research to understand the behaviour of ecological indicators, in view of
38 their crucial importance for the management and protection of marine coastal areas.

39
40 **Keywords:** ecological indicators, benthic macrofauna, intertidal, ecological status,
41 estuaries, Portugal

44 **1. Introduction**

46 **1.1. Background**

47 Ecological indicators are commonly used to provide synoptic information about the
48 state of ecosystems. Most often they summarise information on the structure and/or
49 functioning of ecosystems using a particular biological aspect or component of these
50 ecosystems, such as diversity, productivity or environmental conditions (Salas et al.,

51 2006). In general, the application of ecological indicators is not exempt from criticism.
52 Aggregation of different metrics results in oversimplification of the ecosystem under
53 observation. Moreover, the choice of which ecological indicators to use should follow
54 criteria based on the question we want to address and the theoretical background on
55 which the indicator was created. Before being able to use an indicator it is thus necessary
56 to establish its applicability and robustness for a set of conditions, by testing its
57 consistency with the ecological expectation.

58 The implementation of the European Water Framework Directive (WFD) (EC, 2000),
59 as well as the recent European Marine Strategy Directive 2008/56/EC (EC, 2008) require
60 the use of well-founded ecological indicators, which has contributed greatly to develop this
61 research field and stimulate analyses to establish indicators' applicability to a variety of
62 ecosystems. Indeed, since 2000, numerous ecological quality assessment tools have been
63 proposed by the European scientific community (e.g. Borja et al., 2004; Rosenberg et al.,
64 2004; Bald et al., 2005; Simboura et al., 2005; Devlin et al., 2007; Muxica et al., 2007;
65 Pinto et al., 2009). The WFD suggests that the water body ecological quality should be
66 primarily determined by biological quality elements and then by hydromorphological and
67 physicochemical quality elements.

68 The composition and structure of benthic macrofauna is one of the indicated
69 biological quality elements to be used in transitional and coastal waters for quality status
70 assessment. Benthic communities may detect the level of stress locally and integrate the
71 recent history of stress, constituting a sort of memory for the system. Macrofauna are
72 dominated by species with different mobility, life-cycle and tolerance to stress (Dauer,
73 1993), which covers the WFD demand of integrating differently sensitive species. The
74 response of macrobenthic communities to several types of stress is well studied, based on
75 multivariate analyses that take into account variations in species diversity and their relative

76 abundance between perturbed and control sites (e.g. Pearson and Rosenberg, 1978,
77 Warwick and Clarke 1993, Gray 2002). Based on this knowledge it is possible to
78 determine *a priori* the ecological indicator behaviour and thus its appropriateness to detect
79 changes in the variable of interest. The aim of our study was to test the performance and
80 robustness of a large set of ecological indicators based on the composition and structure
81 of macrobenthic communities.

82 A plethora of methodologies with hundreds of indices, metrics and evaluation tools
83 are presently available (Borja & Dauer, 2008; e.g. see summary in Diaz et al., 2004). An
84 ecologically parsimonious approach dictates that investigation should place greater
85 emphasis on evaluating the suitability of indices that already exist prior to developing new
86 ones (Díaz et al., 2004). We thus selected a large set of ecological indicators of different
87 types (Margalef, Shannon-Wiener, Berger-Parker, Taxonomic Distinctness measures,
88 AZTI's Marine Biotic Index (AMBI), Word Infaunal Trophic Index (ITI), and Eco-Exergy
89 based indices) and compared the status of two sites "Z. *noltii* site" and a "bare sediment"
90 in the Mondego estuary (western coast of Portugal; 40°08'N, 8°50'W) over a period of
91 seventeen years. During this period, the estuary experienced changes in the type and
92 severity of anthropogenic stress, which changed macrophyte distribution and macrofauna
93 communities. We asked whether different ecological indicators were able to detect
94 changes and whether these changes were in agreement with the differences observed
95 analysing species composition of macrobenthic assemblages. Specifically, we tested for
96 differences in the macrofauna composition and biological indicators between the periods
97 during and after the major anthropogenic changes. We also assessed which ecological
98 indicators were more robust in detecting these changes.

99

100 **1.2. Synopsis of human induced changes in the Mondego estuary ecosystem**

101 The Mondego estuary is a relatively small (860 ha) warm-temperate intertidal system.
102 At present, the entire catchment area contributes a large loading of nutrients and several
103 chemicals into the system. Moreover, the estuary supports industrial activities, salt-
104 extraction, and aquaculture farms, and is located close to a tourism centre (Figueira da Foz).
105 The estuary's terminal part is 7 km long and is 2-3 km across at its widest part, consisting
106 of two arms, separated by the Murraceira Island (Fig. 1). The northern arm is deeper (5-10
107 m during high tide), constituting the main navigation channel and the harbour location. The
108 southern arm is shallower (2-4 m during high tide), and is characterised by large areas of
109 intertidal flats exposed during low tide (almost 75% of the total area of the southern arm).
110 A detailed description of the system can be found in Cardoso et al. (2004) and Marques et
111 al. (2007). A comprehensive study on the environmental quality of the Mondego estuary was
112 carried out during the last two decades (Marques et al., 2007) and covered several quality
113 elements (water quality, hydraulics and sediments dynamics, benthic intertidal and subtidal
114 communities, *Z. noltii* beds and green macroalgal cover). Such analyses allowed detection
115 increased eutrophication symptoms and ecological deterioration in the South arm since early
116 1990s, when the communication between the two arms was interrupted, increasing water
117 residence time and nutrient concentration (Lillebø et al., 2005).

118 Since then, macroalgal blooms became more frequent and intense (mostly *Ulva* spp.)
119 (e.g. Martins et al., 2001; Cardoso et al., 2004; Pardal et al., 2004; Leston et al., 2008) and
120 have affected *Z. noltii* beds, reducing the area covered by the seagrass and the biodiversity
121 and community biomass in the remaining meadow (Cardoso et al., 2008; Lillebø et al.,
122 2005). Before the interruption, the *Z. noltii* covered 15 ha (in 1986), which decreased to
123 1.6 ha in 1993, when macroalgal blooms increased up to 415 g m² (Fig. 2). In 1997 *Z.*
124 *noltii* bed reached its lowest value (0.02 ha). Through time, such modifications promoted
125 the emergence of a simplified trophic structure, which has been analysed in the existing

126 literature (e.g. Dolbeth et al., 2007; Martins et al., 2005; Lopes et al., 2006; Patrício and
127 Marques, 2006). Since 1998, the system has started recovering, after the decrease in
128 freshwater discharge from the Pranto River and the limited re-establishment of
129 communication between the two arms (Marques et al., 2007; Leston et al., 2008; Cardoso
130 et al., 2008). *Z. noltii* meadow has begun to recover and in 2005, it occupied an area of
131 about 4.2 ha.

132

133

134 **2. Material and Methods**

135

136 **2.1. Description of the database used**

137 Intertidal benthic communities were first sampled in 1985 (during winter) and 1986
138 (during summer). Subsequently, the community was regularly monitored from January
139 1993 to January 1996, and again from January 1999 to December 2002 once every two
140 weeks from 1993 to 1996, and monthly from 1999 to 2002.

141 In all cases, sampling was conducted at two sites in the South arm (Fig. 1), within *Z.*
142 *noltii* meadow, which persisted during early 1990s, and on an area where the *Z. noltii* has
143 disappeared, now characterised by bare sediment. Each time and at each site, six
144 replicate cores were taken to a depth of 20 cm (13 cm inner diameter and then pooled
145 together for macrofauna analyses. Sediment was washed through a 500 µm mesh sieve
146 and animals preserved in 4% buffered formalin. Animals were identified to species level
147 and subsequently dried at 70°C for 72h to estimate biomass as dry weight (DW) and ash
148 free dry weight (AFDW), after combusting samples for 8h at 450°C.

149

150 **2.2. Hydrological changes and variation in macroalgal and macrophyte coverage**
151 **and biomass**

152 Hydrologic changes in the Mondego estuary, following human interventions, have
153 been assessed using technical reports, maps and aerial photographs. Samples of
154 macrophytes and macroalgae were taken once every two weeks from January 1993 to
155 January 1996, and subsequently, from January 1999 to December 2002, with a monthly
156 periodicity. Six cores (13 cm diameter to a depth of 20 cm) were randomly taken from each
157 site (*Z. noltii* site and bare sediment site), during low tide. Macroalgal and macrophyte
158 species were identified to the species' level, dried at 70°C for 48 hours and then
159 combusted at 450°C for 8 hours to estimate biomass as AFDW. Additionally, the area of *Z.*
160 *noltii* meadows was measured through aerial photographs and field campaigns (Marques
161 et al., 2007).

162
163 **2.3. Ecological indicators**

164 **2.3.1. Description and calculation**

165 The following ecological indicators were calculated using the macrofauna database,
166 for each site and time. Replicates within site and time were pooled.

- 167 1. The **AZTI's Marine Biotic Index, AMBI**, (Borja et al., 2000) is based on the proportion
168 of five ecological groups to which the benthic species are allocated. The soft bottom
169 macrofauna was divided into five groups according to their sensitivity as a function of
170 an increasing stress (see Table 1 for details). The AMBI 4.0 software, freely available
171 at <http://www.azti.es>, was used to calculate this index, using a species list of July 2006
172 and following Borja and Muxika (2005) guidelines. AMBI ranges from 0 to 7, and a
173 better quality trend would be indicated by a decrease on the index value (see Table 1).

- 174 2. To estimate Taxonomic Diversity indices – **Taxonomic Diversity**, Δ ; **Taxonomic**
175 **Distinctness**, Δ^* and **Total Taxonomic Distinctness**, $s\Delta^+$ – a hierarchical Linnean
176 classification was used as a proxy for cladograms representing the relatedness of
177 individual species. For each location, a composite taxonomy was compiled, five
178 taxonomic levels were considered (species, genus, family, order, class and phylum)
179 (Clarke and Warwick, 1998, 2001a) and the indices were calculated using PRIMER 5
180 (Software package from Plymouth Marine Laboratory, UK) (Table 1). Higher values of
181 these indices suggest that, on average, the species in the assemblage are not closely
182 related, being higher biodiversity.
- 183 3. The **Berger-Parker**, **Margalef** and **Shannon-Wiener** indices were also calculated
184 (see Table 1 for details). The Berger-Parker takes values from 0 to 1, with higher
185 values corresponding to lower diversity. An increase on Margalef index is generally
186 accepted as an indication of quality improvement. The \log_2 was used to compute the
187 Shannon-Wiener; therefore the results were expressed in bits/individual and higher
188 values correspond to higher diversity.
- 189 4. The **Infaunal Trophic Index**, **ITI**, was calculated following Codling and Ashley (1992).
190 For each sample, the taxa contributing to the top 80% of the abundance have been
191 allocated to trophic groups. In addition, in samples with ten or less identified taxa, all
192 were taken into consideration for calculation purposes. Once generated, the list of the
193 unclassified taxa was compared with the lists provided by Word (1979, 1990). Table 2
194 shows the Mondego estuary species' trophic classification. Values of the index vary
195 from 0 to 100 with low values indicating degraded conditions. ITI values were used to
196 classify areas of seabed into either "normal" (ITI values 100-60), "modified" (60-30) or
197 "degraded" (30-0) (Bascom et al., 1978).

198 5. **Exergy** expresses the work capacity relative to the same system but at
199 thermodynamic equilibrium with its environment, which means that all the components
200 would be inorganic at the highest possible oxidation state and homogeneously
201 distributed at random in the system (no gradients). Of course exergy cannot be
202 measured precisely, as the total internal energy content of a body cannot be measured
203 exactly. Nevertheless, with regard to far from equilibrium systems (e.g. ecosystems), a
204 reasonable approximation can be obtained if exergy is calculated only from the
205 chemical potentials (which are in fact extremely dominant in ecosystems) (Jørgensen,
206 2000). This approximate measure can be called Eco-Exergy index (Marques et al.,
207 1997). If the total biomass in the system remains constant through time, then the
208 variation of Eco-Exergy will be a function of only the quality of the biomass (structural
209 complexity) or, in other words, of the information embedded in the biomass, which may
210 be called **Specific Eco-Exergy**. Both indices were calculated using macrofauna
211 biomass data and β -values given by Jørgensen et al. (2005) according to the algorithm
212 shown in Table 1.

213

214 ***2.3.2. Criteria used to assess the ecological indicators' success/failure***

215 According to Borja et al. (2008), index validation should ideally include (1) testing of
216 the index using an independent data set, different from the index development data set; (2)
217 setting *a priori* correct classification criteria and/or (3) presentation of a strong *a posteriori*
218 justification for use based upon best professional judgment. The first requirement was
219 fulfilled using our own data set from two intertidal sites in the Mondego estuary. In order to
220 assess the indices success or failure to discriminate the ecological quality status, two main
221 criteria were used:

- 222 1) The classification proposed by the index authors. High values of Margalef, Shannon-
223 Wiener, Taxonomic distinctness indices, ITI and the thermodynamically based indices
224 (TBI) indicate high ecological quality status. High values of Berger-Parker and AMBI
225 indicate low ecological quality status (Table 1).
- 226 2) Determining the level of agreement provided by an index with best professional
227 judgment. In our case study, the indices scores were compare with the existing
228 knowledge about the ecological changes of the system (e.g. Marques et al., 1993,
229 2007; Pardal et al., 2000, 2004; Dolbeth et al., 2007; Cardoso et al., 2004, 2008;
230 Lillebø et al., 2005, 2007; and the present study) according to ultimate causal
231 stressors that had induced such changes (e.g. hydrology and eutrophication) and to
232 other potential stressors (proximate causes) generated by changes in hydrology and
233 eutrophication (e.g. changes in seagrass cover and biomass (*Z. noltii* site); changes in
234 the algal mats biomass (bare sediment site); nutrients levels; sediment grain size and
235 total organic carbon (TOC).

236

237

238 **2.4. Statistical analysis**

239 **2.4.1. Univariate analysis**

240 One-way ANOVA, with time as fixed factor (2 levels: “during the interruption of flow
241 between the North and South arms – 1993 to 1995” and “after the experimental opening of
242 the connection between the two arms of the estuary- 2000 to 2002”) and 30 and 29
243 replicates for *Z. noltii* site and bare sediment site, respectively, was used to test for
244 temporal differences in each of the two sites (with respect to total abundance, total
245 biomass, number of species, Margalef, Shannon-Wiener, Berger–Parker, Biotic Coefficient
246 (AMBI), Taxonomic Distinctness indices, ITI, Eco-Exergy and Specific Eco-Exergy). The

247 “before the interruption- 1985-1986” period was left out from this analysis due to its small
248 number of replicates. Data were checked for normality using the Kolmogorov-Smirnov test
249 and homogeneity of variances was assessed by the Levene’s test. Data were transformed
250 when homoscedasticity assumption was not met. The analyses were performed using the
251 software package Minitab version 12.2. In each site, we expected no differences during
252 the years of interruption, which strongly increased eutrophication effects and
253 sedimentation, thus affecting *Z. noltii* and the post-management years.

254

255 **2.4.2. Multivariate analysis**

256 In order to examine the benthic macroinvertebrate communities’ temporal dynamics
257 in Mondego estuary South arm, a multivariate analysis was performed on biological data
258 from the *Z. noltii* site and bare sediment site using the PRIMER 5.2.6 © software (Software
259 package from Plymouth Marine Laboratory, UK). Data of abundance were square root
260 transformed prior to analysis in order to scale down the effects on the ordination of highly
261 abundant species (Clarke and Warwick, 2001b). The Bray Curtis similarity matrix was
262 calculated and used to generate a 2-dimensional plot with the non-metric multidimensional
263 scaling (nMDS) technique (Clarke, 1993). The one-way ANOSIM test was performed to
264 test for differences among time periods. Species contributing 70% to differences between
265 periods were identified using similarity percentages (SIMPER; Clarke, 1993).

266

267

268 **3. Results**

269

270 **3.1. Changes in macrofauna assemblages**

271 In both sites, there was a significant decline in total macrofauna abundance in the
272 “after the experimental opening” period (1-way ANOVA, *Z. noltii* site: $F_{1,58} = 12.59$, $p <$
273 0.01 ; bare sediment site: $F_{1,56} = 24.06$, $p < 0.01$) (in seagrass bed, from 1993 to 1995:
274 35181 - 249966 ind m^{-2} ; from 2000 to 2002: 5785 - 120375 ind m^{-2} ; in the bare sediment
275 site, from 1993 to 1995: 6515 - 167554 ind m^{-2} ; from 2000 to 2002: 3430 - 28324 ind m^{-2})
276 (Fig. 3A). Whereas the total biomass, in the *Z. noltii* site, was significantly higher “during
277 the interruption” period (1-way ANOVA, $F_{1,58} = 7.63$, $p < 0.01$), ranging from 58 to 158 g m^{-2} .
278 In the bare sediment site, the total biomass was significantly higher after the
279 implementation of preliminary management measures (range 20-75 g m^{-2}) (1-way ANOVA,
280 $F_{1,56} = 20.59$, $p < 0.01$) (Fig. 3B). In both sites, more species were present “during the
281 interruption” period compared to “after the experimental opening” period. (Fig. 3C) (1-way
282 ANOVA, *Z.noltii* site: $F_{1,58} = 77.23$, $p < 0.01$; bare sediment site: $F_{1,56} = 15.65$, $p < 0.01$).
283 Generally, the *Z. noltii* site supported more species than the bare sediment site (in the
284 *Z.noltii* site, from 1993 to 1995: 14-40 species; from 2000 to 2002: 9-25 species; in the
285 bare sediment site, from 1993 to 1995: 7-28 species; from 2000 to 2002: 7-20 species).

286 In both sampling sites, the results of the one-way ANOSIM test showed significant
287 differences ($p < 0.001$) between the two periods. Nonetheless, the *Z. noltii* site had higher
288 Global *R* value than the bare sediment site (Global *R* = 0.42 and 0.32, respectively). The
289 nMDS showed that the “after the experimental opening” period is closer to the reference
290 points, particularly in the *Z. noltii* site (Fig. 4).

291 The SIMPER analysis showed that in the *Z. noltii* site, *Hediste diversicolor*, *Hydrobia*
292 *ulvae*, *Scrobicularia plana*, *Cyathura carinata*, Tubificidae, *Alkmaria romijni*,
293 *Heterosmastus filiformis*, *Capitela capitata*, *Cerastoderma edule* and *Chaetozone setosa*
294 were the species responsible for the separation between periods (Table 3). During the
295 period of works and closure of the channel, the abundance of some polychaete species

296 (*A. romijni*, *H. filiformis*, *C. capitata* and *C. setose*), of Tubificidae and *H. ulvae*
297 dramatically increased as compared to the period before disturbance (1986) and also to
298 the period following mitigation measures (1998-2002) (Table 3).

299

300 **3.2. Ecological indicators**

301 In both sites, Margalef (Fig. 5A) exhibited significant differences between the two
302 time periods considered (1-way ANOVA, *Z. noltii* site: $F_{1,58} = 58.70$, $p < 0.01$; bare
303 sediment site: $F_{1,56} = 12.29$, $p < 0.01$), presenting unexpected higher values “during the
304 interruption” period. Overall, Margalef was always higher in the *Z. noltii* site than the bare
305 sediment site. The qualitative measure based on the presence/absence of species, TTD
306 (Fig. 5B), showed similar behaviour (1-way ANOVA, *Z. noltii* site: $F_{1,58} = 76.82$, $p < 0.01$;
307 bare sediment site: $F_{1,56} = 15.12$, $p < 0.01$).

308 In contrast to the diversity indices of Margalef and TTD, there were no difference in
309 the Taxonomic Distinctness between periods (Fig. 5C), ($p > 0.05$), and the index was not
310 larger in the *Z. noltii* compared to the bare sediment site.

311 The Taxonomic Diversity (Fig. 5D) and ITI (Fig. 5E) were unable to detect temporal
312 changes in the *Z. noltii* site, but, as theoretically expected, exhibited lower values “during
313 the interruption” period at the bare sediment site (1-way ANOVA, $F_{1,56} = 10.67$, $p < 0.01$
314 and $F_{1,56} = 6.49$, $p < 0.01$, respectively). Both indices were lower at *Z. noltii* site, differing
315 from the behaviour that we could predict based on the authors classification and our own
316 knowledge regarding the system. According to Bascom et al. (1978), ITI classified the
317 benthic community in the bare sediment site community as a “slightly modified” to
318 “normal”.

319 The Shannon-Wiener (Fig. 5F) values were higher in the bare sediment site, because
320 of the dominance of *H. ulvae* in the *Z. noltii* site (almost 90% of the total abundance).

321 While no significant differences were detected between the pre- and post-management
322 periods in the reference site (1-way ANOVA, $F_{1,58} = 0.14$, $p = 0.71$), in the bare sediment
323 site, the values were significantly higher “after the experimental opening” period (1-way
324 ANOVA, $F_{1,56} = 9.68$, $p < 0.01$). As expected, the Berger-Parker dominance (Fig. 5G)
325 showed the opposite behaviour, with higher values in the *Z. noltii* site, again due to *H.*
326 *ulvae*'s dominance. In the bare sediment site, the dominance significantly decreased after
327 the implementation of the management plan (1-way ANOVA, $F_{1,56} = 12.82$, $p < 0.01$).

328 A significantly higher AMBI value (Fig. 5H) was found “during the interruption” period
329 at the reference site (1-way ANOVA, $F_{1,58} = 5.91$, $p < 0.02$), indicating worse ecological
330 conditions during this time period. In the bare sediment site, no differences regarding the
331 pre- or post restoration periods were detected (1-way ANOVA, $F_{1,56} = 2.53$, $p > 0.05$). This
332 index showed similar values in both sites.

333 In the *Z. noltii* site, Eco-Exergy and Specific Eco-Exergy (Fig. 5I and J) were
334 significantly higher “during the interruption” period (1-way ANOVA, Eco-Exergy: $F_{1,58} =$
335 8.66 , $p < 0.01$; Specific Eco-Exergy: $F_{1,58} = 56.85$, $p < 0.01$). In the bare sediment site,
336 these indices showed the opposite behaviour, with significantly higher values “after the
337 experimental opening” period (1-way ANOVA, Eco-Exergy: $F_{1,56} = 25.24$, $p < 0.01$; Specific
338 Eco-Exergy: $F_{1,56} = 4.8$, $p < 0.03$), indicating higher biomass structure and information
339 embedded in it. The indices had higher values in the *Z. noltii* site than in the bare sediment
340 site, following the behaviour that we would predict based on *a priori* established criteria.

341

342

343 4. Discussion

344

345 4.1. Ecological indicators performance

346 Our analyses on the long-term changes in the composition of macrofauna
347 assemblages confirmed the previous studies (e.g. Dolbeth et al., 2007; Marques et al.,
348 2007; Cardoso et al., 2008) and highlighted that the system seems to be recovering from
349 the environmental changes occurred during the 1990s. At both sites, during the 1993-
350 1995, the total dissolved inorganic nitrogen in the water column was higher than “after the
351 experimental opening period” (Leston et al., 2008), the macroalgae assemblages were
352 dominated by Ulvaceans, the *Z. noltii* cover and biomass reached their lowest values
353 (Marques et al., 2007) and the abundance of the opportunistic species increased. After
354 1997/1998, the macrofauna and macrophyte assemblages showed a partial recovery
355 towards the reference situation.

356 The selected ecological indicators are based upon community characteristics that
357 are typically part of, or inherent to, the diversity of definitions of biotic integrity that include
358 elements of species diversity, abundance, energy-flow-food web structure, maintenance of
359 complexity and self-organization (Borja and Dauer, 2008). Therefore, according to the
360 classification proposed, we would expect significantly higher values for Margalef,
361 Shannon-Wiener, Taxonomic Distinctness measures, ITI, Eco-Exergy and Specific Eco-
362 Exergy; AMBI and Berger-Parker should present significant lower values during the pos-
363 management period, at both sites. In the *Z. noltii* site, only AMBI reflected the ecological
364 expectation, of a community with higher abundance of opportunist species “during the
365 interruption” period. Margalef, TTD and the TBI indicated higher diversity and biomass
366 structure/information “during the interruption” period, indicating that this community was
367 apparently able to obtain some benefit from the existing conditions (higher nutrient input,
368 lower water residence time). In the bare sediment site, with the exception of Margalef and
369 TTD, all ecological indicators able to detect significant differences between time periods,

370 performed as initially projected, indicating a better ecological status “after the experimental
371 opening” period.

372 Furthermore, even if hydrology and eutrophication (both water column related), were
373 probably the major drivers “shaping” South arm environmental conditions, the two study
374 sites were differently affected by distinct stressors resulting from these drivers. The bare
375 sediment site, characterised by sandy sediments, which have not supported rooted
376 macrophytes for more than 15 years, lower organic matter content (mean $3.0\% \pm 1.14$) and
377 lower water-flows ($0.8\text{-}1.2\text{ m s}^{-1}$) has been regularly covered by extensive algal mats (e.g.
378 Lillebø et al., 2005; Dolbeth et al., 2007; Marques et al., 1997, 2003, 2007; Cardoso et al.,
379 2008). These macroalgal blooms have well-documented effects, causing dramatic
380 changes in sediment chemistry, nutrient release, rates of oxygen consumption and
381 accumulation of metal sulfides and H_2S in sediment pore waters. These shifts are
382 profound changes in habitat quality for invertebrate assemblages (e.g. Pearson &
383 Rosenberg, 1978; Raffaelli et al 1998; Cloern, 2001; Patrício & Marques, 2006). On the
384 other hand, the *Z. noltii* site, represents a distinct complex and heterogeneous habitat with
385 particular physical and chemical conditions, characterised by muddy sediments covered
386 with rooted macrophytes, higher organic matter content (mean $6.2\% \pm 1.76$), higher water-
387 flow velocity ($1.2\text{-}1.4\text{ m s}^{-1}$) (e.g. Dolbeth et al., 2007) and supporting a higher diversity of
388 species (Cardoso, 2004). Knowing that the direct and indirect responses to changes in
389 hydrology and nutrient enrichment are expressed in varying degrees in different habitats
390 (Cloern, 2001), and taking into consideration the effect of site-specific operative stressors,
391 we have considered *Z. noltii* site as representing a better ecological condition. Therefore,
392 we would expect higher values for Margalef, Shannon-Wiener, Taxonomic Distinctness
393 measures, ITI, Eco-Exergy and Specific Eco-Exergy in the reference site; AMBI and
394 Berger-Parker would present the opposite behaviour. In fact, only Margalef, TTD and TBI

395 followed the behaviour that we could predict based on our knowledge of the system (Fig.
396 4). Taxonomic Distinctness and AMBI presented similar values at both sites, indicating that
397 these two metrics may be of limited efficacy in understanding the environmental conditions
398 being investigated. AMBI values were dominated by *H. ulvae* abundance. As numerous
399 estuarine species, this gastropod belongs to ecological group III, which comprises species
400 tolerant to an excess of organic matter enrichment, a widespread stressor in transitional
401 systems. Such explanation is supported by considering that Shannon-Wiener and Berger-
402 Parker indices indicated that the *Z. noltii* site was of lower quality than the bare sediment.
403 These indices are based on measures of equitability and dominance, and can vary
404 unexpectedly if one species becomes dominant, as is the case of *H. ulvae*. Indeed,
405 excluding *H. ulvae* from the dataset resulted in opposite results. *H. ulvae* is a very mobile
406 species that may migrate during low tide towards areas more protected and with high food
407 availability, as is the case of *Z. noltii* site. Therefore, more attention should be paid to the
408 ecology and distribution of species to decrease the probability of giving an erroneous
409 classification based on a species with high mobility. Furthermore, it should be noticed that in
410 estuaries species dominance occurs more often than on coastal systems, even in
411 relatively pristine conditions, due the large natural variability of environmental parameters.
412 According to Constanza et al. (1992) and Elliot and Quintino (2007), in these ecosystems,
413 the “positive effect” of natural environmental variability is shown as the ability of some
414 organisms, which can tolerate the adverse and variable environmental conditions, to
415 capitalise on the lack of inter-specific competition and thus achieve high population
416 densities. Therefore, caution should be taken when interpreting equitability and dominance
417 results based in estuarine macrobenthic assemblages.

418 Following Word (1990), ITI showed a higher value in the bare sediment site, where
419 the sediment organic matter content was lower. However, in the *Z. noltii* site the sediment

420 organic matter has a natural source (macrophyte primary production). For that reason, the
421 metric is not correctly capturing the environmental conditions being investigated.
422 Moreover, ITI is difficult to apply in environmental studies once determining the
423 organisms' diet may become reasonably problematic. Generally, the real diet (studied
424 observing stomach content) is difficult to establish, and can vary from one population to
425 another among the same taxonomic entity (e.g. *H. filiformis* was classified by Brown
426 (1985) as subsurface deposit feeder and as surface detritus feeder by Word (1990)).

427 According to our study, it seems that the ecological indicators response was not
428 always temporally and spatially consistent (Table 4). These results are quite interesting,
429 raising further questions.

430 It is well known, that estuaries are particularly challenging due to strong spatial,
431 seasonal and inter-annual variations of environmental characteristics that influence benthic
432 communities (Chainho et al., 2007). Apart from salinity regime, plant presence/absence,
433 sediment grain size and organic matter content, the hydrological conditions, such as flood
434 and drought periods, may have great impact in communities and thus on diversity
435 measures (Pearson and Rosenberg, 1978; Gray, 1997; Raffaelli et al., 1998; Cloern, 2001;
436 Salas et al., 2006; Chainho et al., 2007; Dolbeth et al., 2007; Cardoso et al., 2008; Teixeira
437 et al., 2008).

438 In summary, we may say that although hydrology and eutrophication were the major
439 drivers, other ultimate causal factors, probably also played a role during the study period,
440 making it difficult to unambiguously distinguish the anthropogenic disturbance effects from
441 the natural temporal wide-scale variability. In any case, the ecological indicators' results
442 were constrained to the type and magnitude of the drivers' combined effect that occurred
443 at either or both sites during the study period. Therefore, caution must be taken
444 interpreting values isolated from the ecological community context.

445

446

447 **4.2. Scientific implications and limitations of the study**

448 This paper laid out some of the history of anthropogenic modifications and sketched
449 a brief image of the ecological changes over the considered timeframe (derived partially
450 from previously reported literature). In addition, many readers have pondered comparisons
451 among all of the new indices that seem to continuously emerge (e.g. Díaz et al., 2004;
452 Labruno et al., 2005; Aubry & Elliot, 2006; Quintino et al., 2006; Dauvin et al., 2007;
453 Blanchet et al., 2008). The present study compared several ecological indicators (all using
454 benthic macrofauna) to the image of change that can be cast through a set of
455 measurements on alterations in water quality, hydraulic/sediments, benthic invertebrate
456 and plant/algal communities, and spatial distribution. Even if this broad comparison has
457 been made in the past, given the European initiatives to use indices to monitor estuarine
458 system health, we hope this study could be a useful and directly applicable demonstration
459 of the care which must be taken in selecting appropriate indices. Indeed, it is very
460 challenging to see such different predictions from the diverse indices, given that the same
461 data were used (albeit in different ways, but that was the aim). Blanchet et al. (2008),
462 testing five biotic indices (AMBI, BENTIX, Shannon-Wiener diversity, BQI and BOPA) in
463 two semi-enclosed, sheltered coastal ecosystems and in one transitional water body
464 situated along the Western French coast, also found that these five indices rarely agreed
465 with each other, describing very different pictures of the overall ecological quality status of
466 the three study areas. A similar conclusion was made by Chainho et al. (2007) studying
467 the Mondego estuary subtidal assemblages, using four indices (Margalef, Shannon-
468 Wiener, AMBI and W-Statistic).

469 Notwithstanding, it is obvious for us, that the perfect way to compare indices would
470 be to (ideally) set up an ecological situation, in as controlled a manner as possible, in
471 which one knew the “precise, accurate and totally clear answer” already and where strong
472 putative gradients were selected for validation testing (e.g. Borja et al., 2000; Quintino et
473 al., 2006). Nevertheless, the available ecological tools will have to work in “existent”
474 ecosystems and available datasets. In our case study the ecological indicators diagnostic
475 ability was only tested in two sites: a “*Z. noltii* site” and a “bare sediment site” during two
476 different time periods, characterised by different levels of impact. We did not cover the five
477 categories featured within the WFD (bad, poor, moderate, good and high). Therefore, this
478 study only addresses two conditions and for that reason provides a limited assessment.
479 Clearly, with other case-studies, further steps will have to be done to fulfil the WFD
480 requirements. The second phase of the European WFD Intercalibration exercise will
481 certainly be critical, particularly for transitional systems. Even so, it is our conviction that
482 initially the proposed ecological indicators should be able to give the correct assessment in
483 this case, particularly, when some of these metrics are being selected and combined into
484 multimetric indices used to assess the ecological quality status all over Europe with
485 obvious legal implications. We truly agree that without a sound and obvious ecological
486 foundation, an index will not be policy-relevant and therefore difficult to use to make policy
487 choices (Borja and Dauer, 2008), thus the results must be justifiable and scientifically
488 defensible. Experiences gather from different ecosystems, distinct geographical ranges,
489 various hydrological regimes, and diverse pressure types are vital and urgent.

490

491

492 **5. Conclusions**

493 Relevant conclusions can be taken from our study: (1) Taxonomic Distinctness did not
494 detect temporal and spatial changes; (2) ITI did not detect spatial changes largely because
495 estuarine macrofauna shows diet plasticity and is able to adapt its trophic role; (3) The
496 equitability and dominance measures were totally influenced by the presence of a common
497 species with high mobility, (4) The best distinction between sites was given by Margalef,
498 TTD and TBI, (5) The indicators ability to capture temporal changes was different in each
499 site, revealing habitat-dependency and (6) although some ecological indicators have been
500 effective in differentiating the two time periods, not all have behaved consistently with
501 ecological expectation. These considerations are crucial for management, once an
502 indicator is expected to respond to a particular pressure and lead to a human response to
503 reduce environmental impacts through the systems' driving forces, with all the social and
504 economic repercussions of that action.

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506

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517

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- 713 Word, J.Q., 1979. *The Infaunal Trophic Index*. California Coastal Water Research Project Annual
714 Report 19-39.
- 715 Word, J.Q., 1990. *The Infaunal Trophic Index, a functional approach to benthic community*
716 *analyses*. PhD Dissertation, University of Washington, Seattle, Washington, USA.

717 Table 1. Ecological indicators: algorithms and ecological classification.

Indicator	Algorithm/ Explanation	Classification
1. Species as indicators		
AZTI's Marine Biotic Index (Borja et al. 2000)	$AMBI = \frac{\{(0 \times \%GI) + (1.5 \times \%GII) + (3 \times \%GIII) + (4.5 \times \%GIV) + (6 \times \%GV)\}}{100}$ <p>I. Species very sensitive to organic enrichment and present under unpolluted conditions; II. Species indifferent to enrichment, always in low densities with non-significant variations with time; III. Species tolerant to excess of organic matter enrichment; IV. Second-order opportunist species, mainly small sized polychaete; V. First-order opportunist species, essentially deposit-feeders</p>	Normal 0-1.2 Slightly polluted 1.2-3.2 Moderately polluted 3.2-5.0 Highly polluted 5.0-6.0 Azotic 6.0-7.0
2. Diversity		
Average Taxonomic Diversity (Warwick and Clarke 1995)	$\Delta = \left[\sum_{i < j} \omega_{ij} x_i x_j \right] / [n(n-1)/2]$	
Average Taxonomic Distinctness (Warwick and Clarke 1995)	$\Delta^* = \left[\sum_{i < j} \omega_{ij} x_i x_j \right] / \left[\sum \sum \delta_{i < j} x_i x_j \right]$	Higher value, higher biodiversity – on average species in the assemblage are not closely (phylogenetic) related
Total Taxonomic Distinctness (Warwick and Clarke 1995)	$s\Delta^+ = \left[\sum_i \left(\left[\sum_{i \neq j} \omega_{ij} \right] / (s-1) \right) \right]$ <p>x_i: abundance of the i th of S species observed; $n (= \sum_i x_i)$: total number of individuals in the sample; ω_{ij}: "distinctness weight" given to the path length linking species i and j in the taxonomy</p>	
Berger-Parker Index (Berger and Parker 1970)	$D = n_{max} / N$ <p>n_{max}: number of individuals of the most abundant species; N: total number of individuals</p>	Low diversity 1 High diversity 0
Margalef Index (Margalef 1969)	$D = (S - 1) / \ln(N)$ <p>S: number of species found; N: total number of individuals</p>	Low diversity Low value
Shannon-Wiener Index (Shannon and Weaver 1963)	$H' = - \sum p_i \log_2 p_i$ <p>p_i: proportion of abundance of species i in a community were species proportions are $p_1, p_2, p_3 \dots p_n$; S: number of species found</p>	Low diversity 0 High diversity ≥ 5
3. Ecological strategies		
Infaunal Trophic Index (Word 1979)	$ITI = 100 - 100/3 \left(0n_1 + 1n_2 + 2n_3 + 3n_4 \right) / \left(n_1 + n_2 + n_3 + n_4 \right)$ <p>Macrofauna groups: (1) suspension feeders; (2) interface feeders; (3) surface deposit feeders; (4) subsurface deposit feeders</p> <p>n_1, n_2, n_3, n_4: number of individuals in each of the above mentioned groups</p>	Community dominated by: Group 1 >78 Group 2 58-77 Group 3 25-57 Group 4 < 24
4. Thermodynamically oriented		
Eco-Exergy (Mejer and Jørgensen 1979)	$EX = T \times \sum \beta_i \times C_i$ <p>T: absolute temperature; C_i: concentration in the ecosystem of component i (e.g. biomass of a given taxonomic group or functional group); β_i: factor able to express roughly the quantity of information embedded in the genome of the organisms</p>	Higher value, higher biomass structure and information embedded in the biomass
Specific Eco-Exergy (Jørgensen 2000)	$SpEX = Exergy / Total \ biomass$	Higher value, higher information embedded in the biomass

719 Table 2. Trophic classification of taxa contributing the top 80% of the abundance recorded
 720 in the two studied intertidal sites of the Mondego estuary, from 1985 to 2002. Trophic
 721 group assigned according to references. 1: suspension feeders; 2: suspension and
 722 surface-detritus feeders; 3: surface deposit feeders; 4: subsurface deposit feeders.

Trophic Group	Taxa	References	Trophic Group	Taxa	References
1	<i>Cerastoderma</i>	Morton 1983	3	<i>Carcinus maenas</i>	Riera et al. 1999
	<i>Spio</i>	Dauer et al. 1981		<i>Crangon crangon</i>	Ansell et al. 1999
	<i>Scrobicularia plana</i>	Riera et al. 1999		<i>Cyathura carinata</i>	Ferreira et al. 2004
2	<i>Alkmaria romijni</i>	Word 1990 (Ampharetinae)		<i>Hediste diversicolor</i>	Garcia-Arberas & Rallo 2002
	<i>Ampithoe</i>	Dixon & Moore 1997		<i>Hydrobia ulvae</i>	Philippart 1995
	<i>Corophium</i>	Word 1990		Diptera	Livingston 2003
	<i>Chaetozone setosa</i>	Word 1990		<i>Mysta picta</i>	Solis-Weiss et al. 2004
	<i>Heteromastus</i>	Word 1990	4	<i>Capitella</i>	Word 1990
	<i>Mediomastus</i>	Word 1990		Oligochaeta sp1	Word 1990
	<i>Melita palmata</i>	Word 1990		Tetrastemma	Barnes & de Villiers 2000
	<i>Pygospio elegans</i>	Word 1990		Tubificoides	Gaston et al. 1998
	<i>Streblospio shrubsolii</i>	Word 1990			

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Table 3. Average abundance values (ind. m⁻²) of the species that contributed more than

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70% to the difference between periods in the *Z. noltii* and in the bare sediment sites.

	Before the interruption	During the interruption	After the experimental opening
<i>Zostera noltii</i> site			
<i>Hediste diversicolor</i>	1189	19	523
Tubificidae	0	2944	714
<i>Alkmaria romijni</i>	204	993	443
<i>Heterosmastus filiformis</i>	265	550	357
<i>Capitela capitata</i>	114	241	92
<i>Chaetozone setosa</i>	0	1756	31
<i>Hydrobia ulvae</i>	1960	80691	41862
<i>Scrobicularia plana</i>	596	1023	724
<i>Melita palmata</i>	83	336	75
<i>Cyathura carinata</i>	271	217	173
Oligochaete	0	220	100
<i>Streblospio shrubsolii</i>	60	349	138
<i>Cerastoderma edule</i>	13	519	74
Bare sediment site			
<i>Hediste diversicolor</i>	523	50	265
<i>Hydrobia ulvae</i>	1166	27192	5573
<i>Alkmaria romijni</i>	93	6076	1483
<i>Cyathura carinata</i>	638	2043	2177
<i>Scrobicularia plana</i>	319	2347	2763
<i>Amphicteis gunneri</i>	106	0	0

Table 4. Summary of the ecological indicators trends obtained for the *Z. noltii* and bare sediment sites, considering “during the interruption” and “after the experimental opening” (after) periods. In bold are the values that are compliant with *a priori* established classification criteria.

Ecological indicator	Site	
	<i>Z. noltii</i>	Bare sediment
Margalef		
Total Taxonomic Distinctness	↓ after	↓ after
Taxonomic Distinctness	p>0.05	p>0.05
Eco-Exergy		
Specific Eco-Exergy	↓ after	↑ after
Berger-Parker		↓ after
Shannon-Wiener	p>0.05	↑ after
Taxonomic Diversity		
Infaunal Trophic Index	p>0.05	↑ after
AMBI	↓ after	p>0.05

Figures captions

Figure 1. Mondego estuary. Location of the two intertidal sampling sites (black circles): *Z. noltii* site and bare sediment site and position of the connection area between the North and South arms (open circle). NA-north arm, SA-south arm.

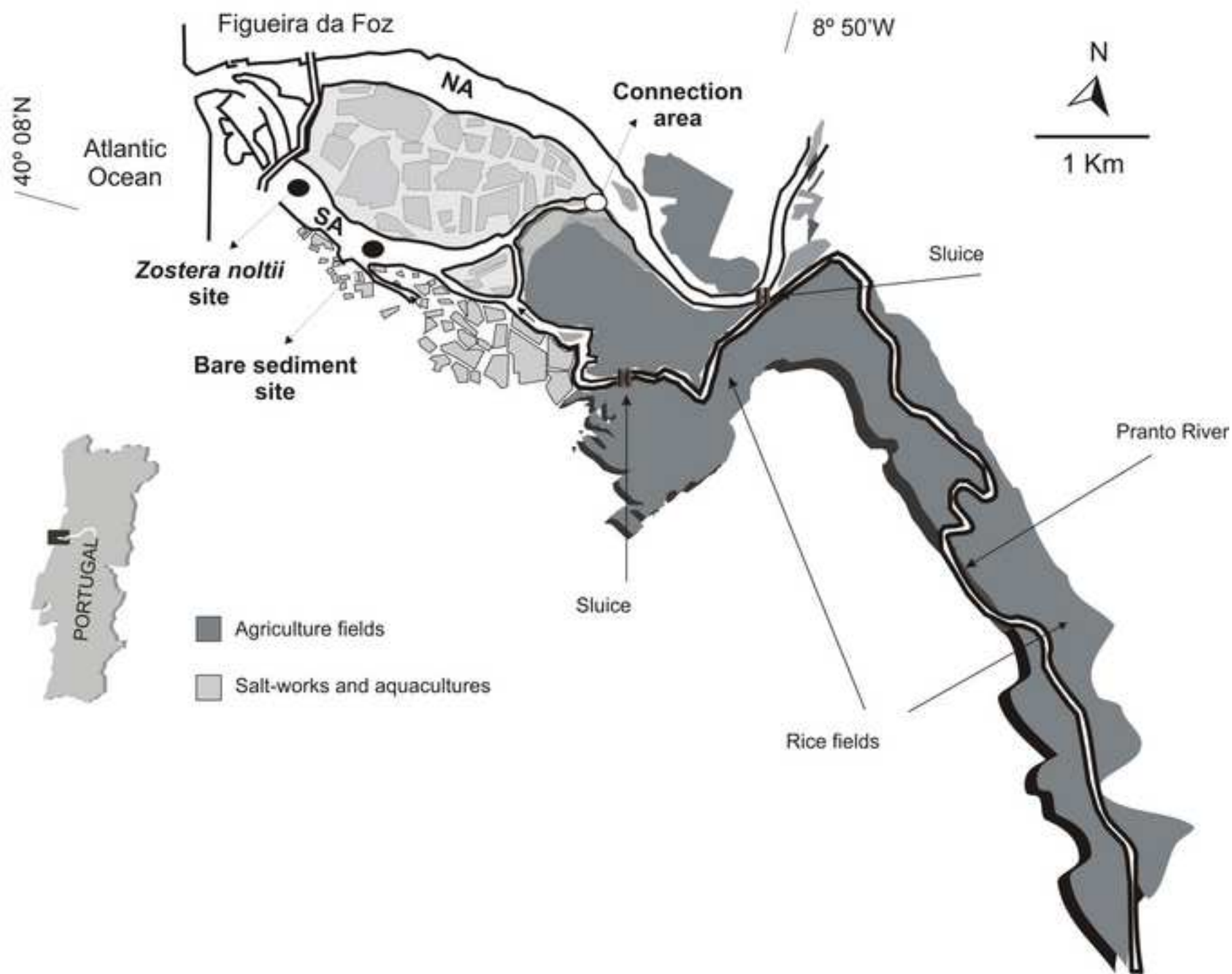
Figure 2. Temporal changes of *Z. noltii* meadows' area (ha) and biomass (g AFWD m⁻²), green macroalgae biomass (g AFWD m⁻²) and the connection width between the two estuarine arms. Black areas correspond to *Z. noltii* site, bare sediment site and connection between the two arms. NA-north arm, SA-south arm.

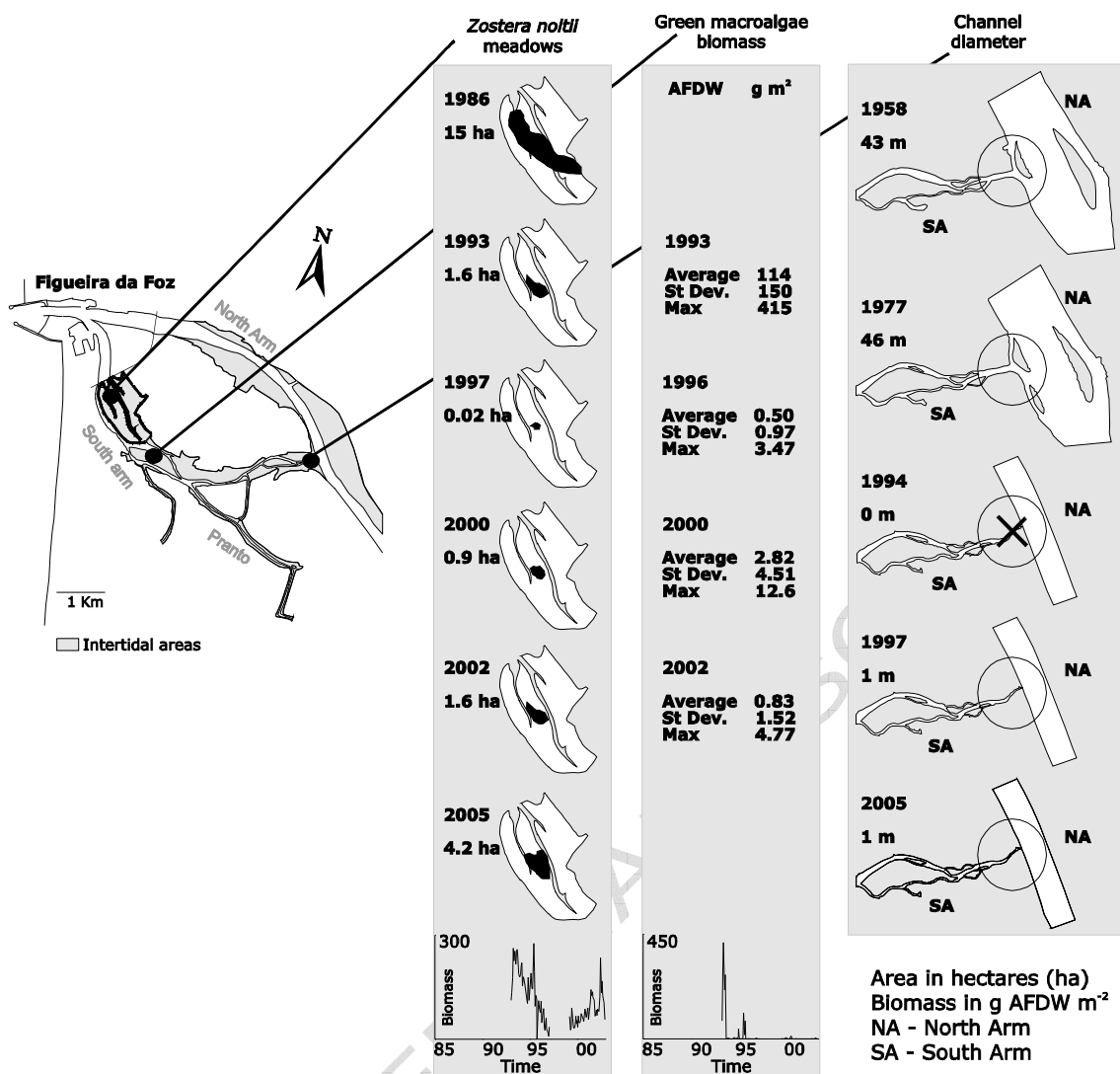
Figure 3. Macrofauna A. total abundance, B. total biomass and C. number of species in the *Z. noltii* site and in the bare sediment site, during the study period.

Figure 4. A Two-dimensional, non-metric, multidimensional scaling (nMDS) ordination based on abundance data. A. *Z. noltii* site and B. bare sediment site, for the three time periods: before the perturbation under assessment (1985 and 1986), period without connection between arms (1993, 1994 and 1995) and after experimental mitigation measures (1999-2002). Each point represents a different time period at the same site. Stress values are shown for each nMDS plot indicating the similarity between the multi-dimensional representation and the 2-dimension plot.

Figure 5. Temporal and spatial variation of different ecological indicators applied to data of the intertidal communities of the South arm of the Mondego estuary, during the study period.

Figure





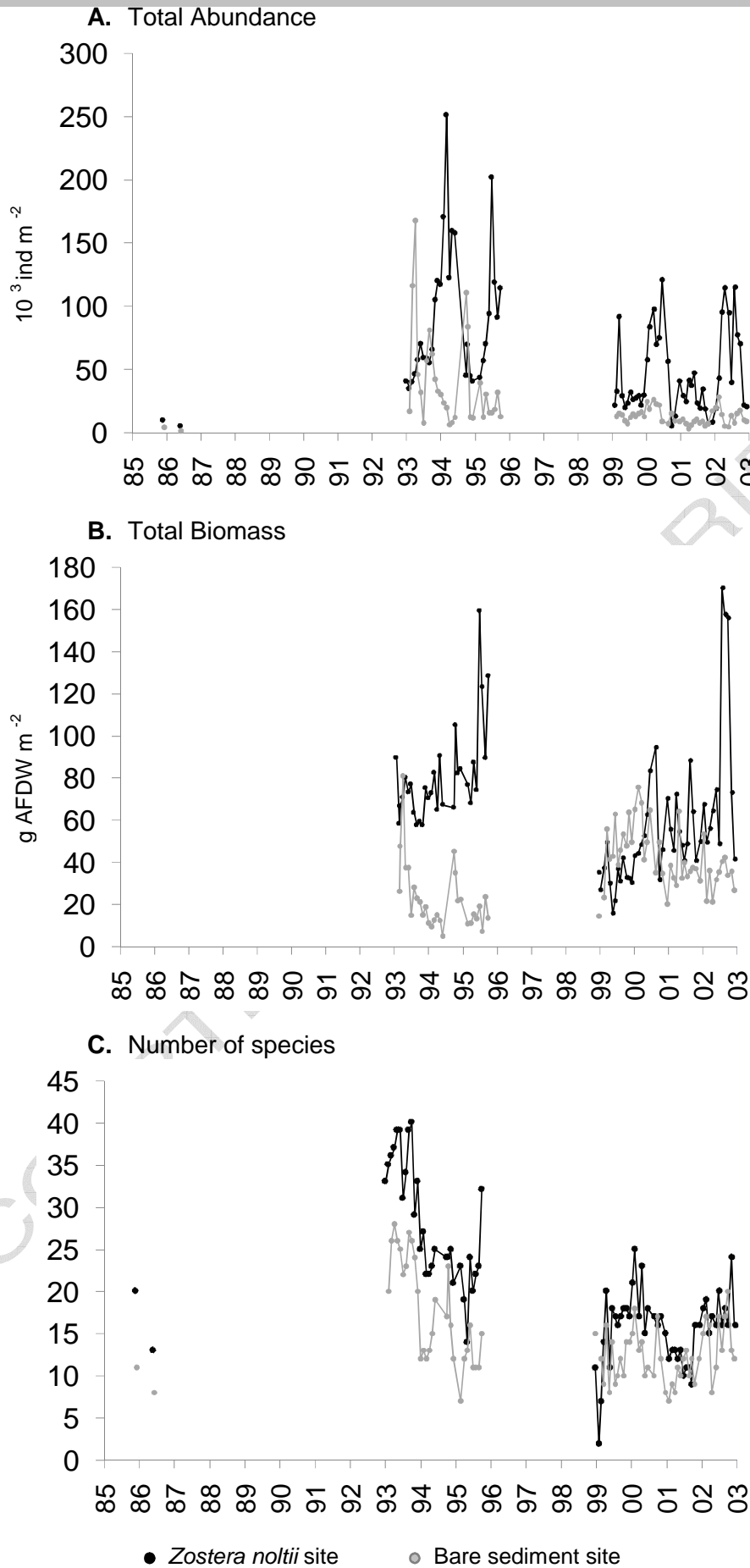
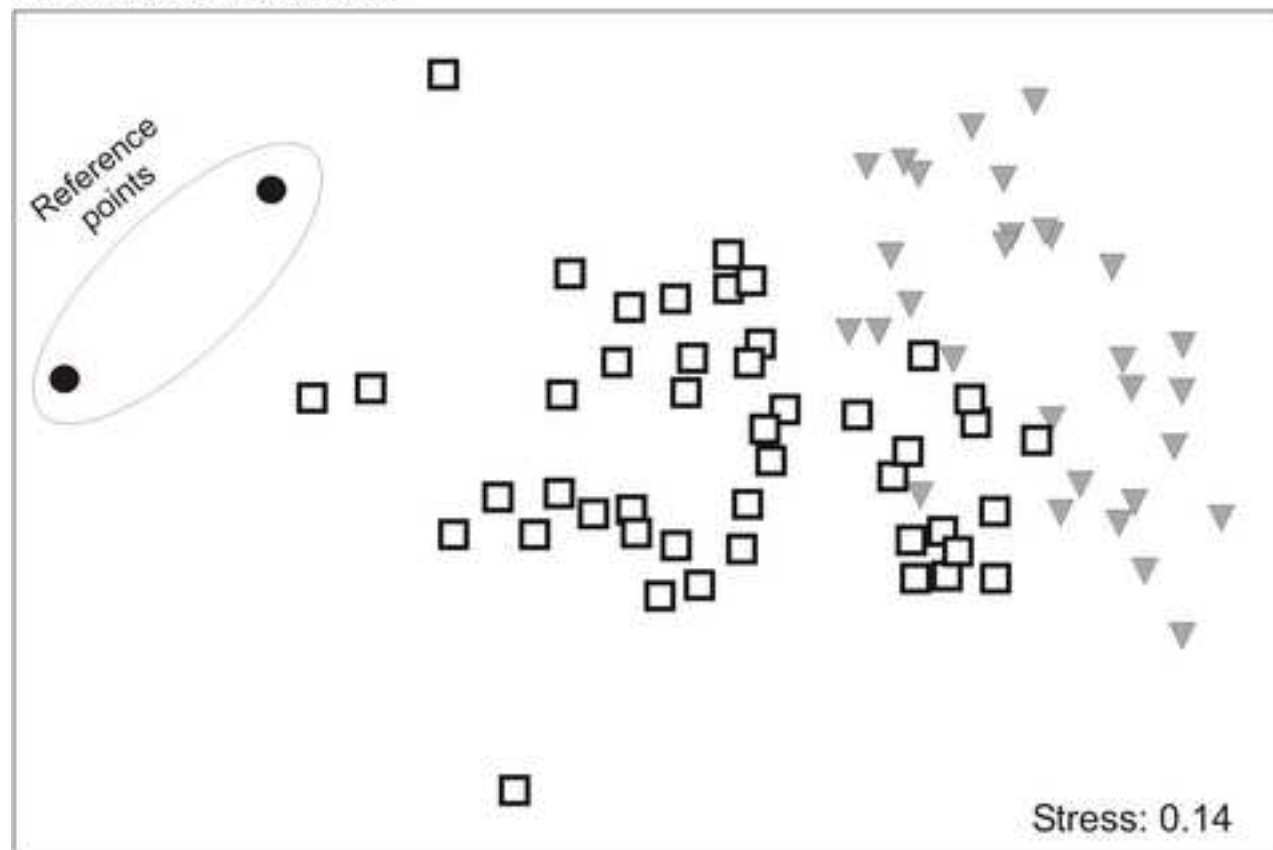
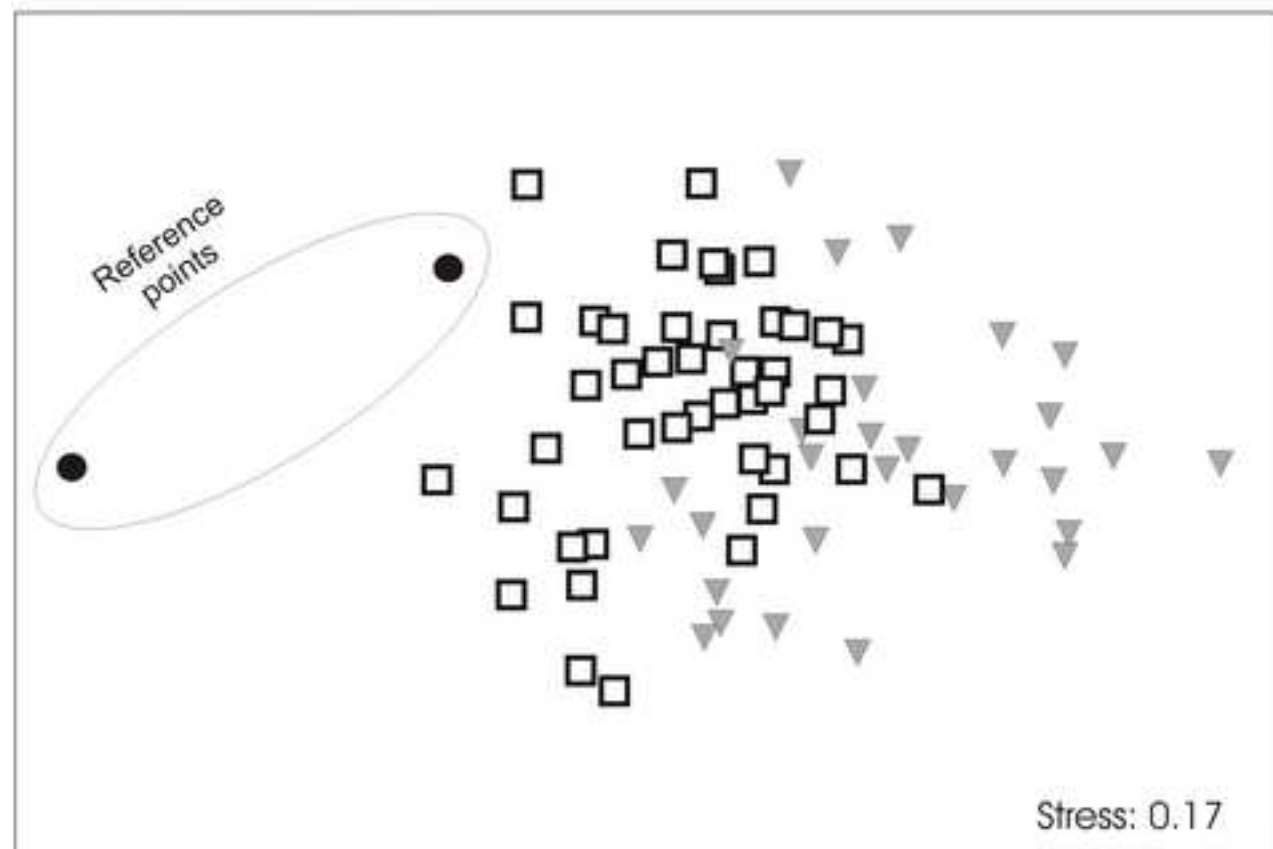


Figure 3

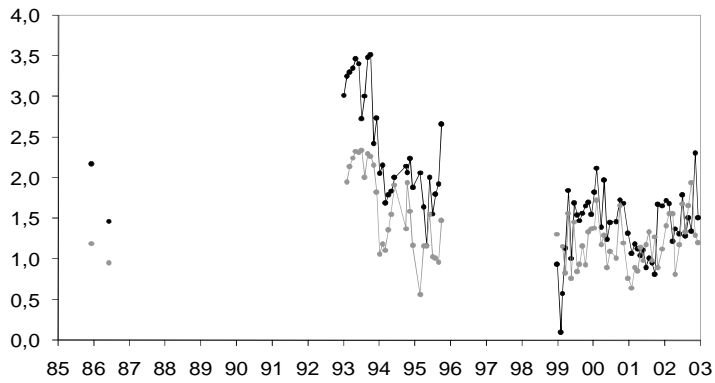
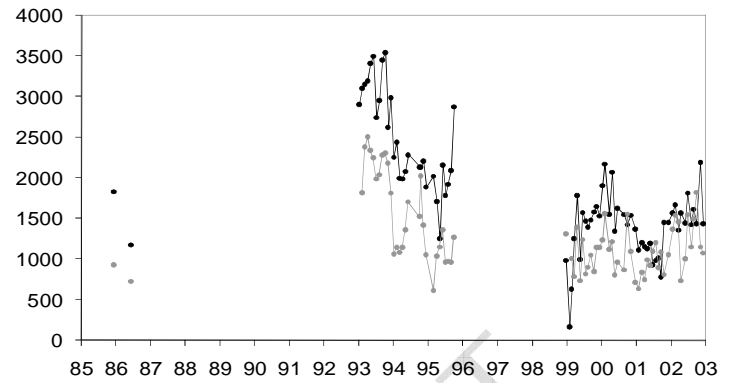
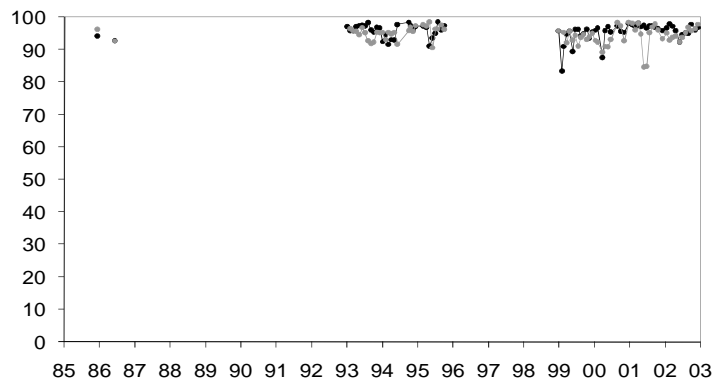
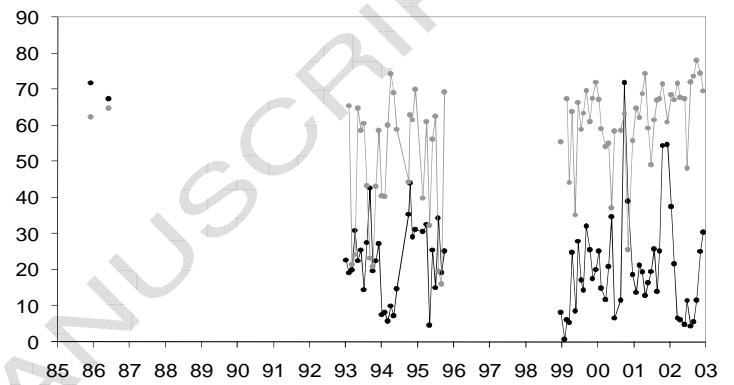
A. *Zostera noltii* site**B. Bare sediment site**

● Before perturbation

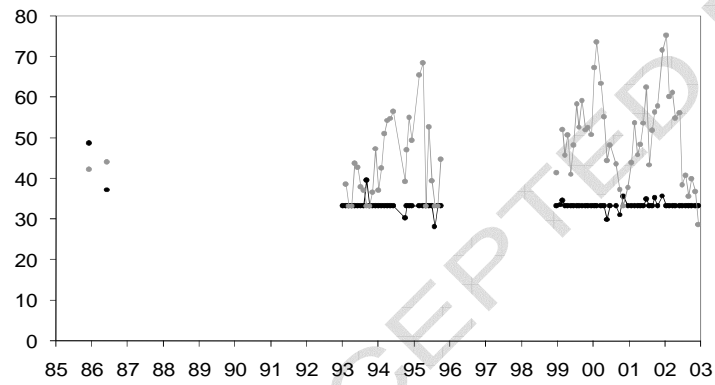
▼ During interruption

□ After experimental opening

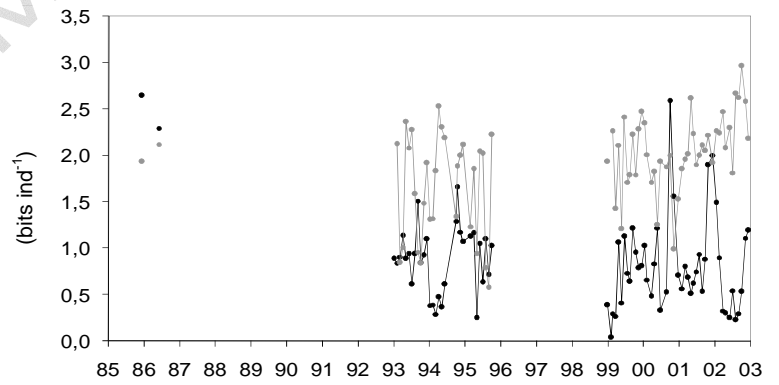
A. Margalef

B. Total Taxonomic Distinctness, $s\Delta^+$ C. Taxonomic Distinctness, Δ^* D. Taxonomic Diversity, Δ 

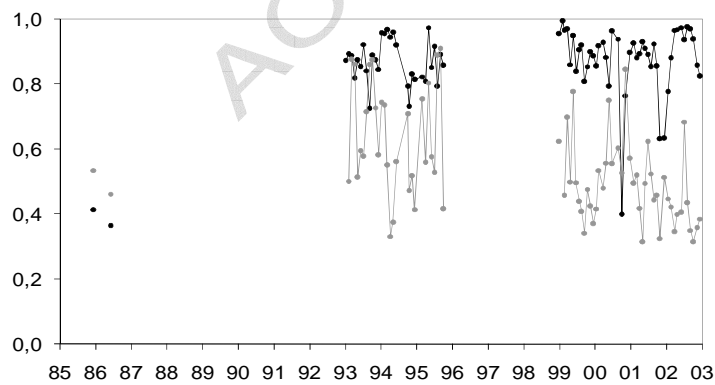
E. Infaunal Trophic Index



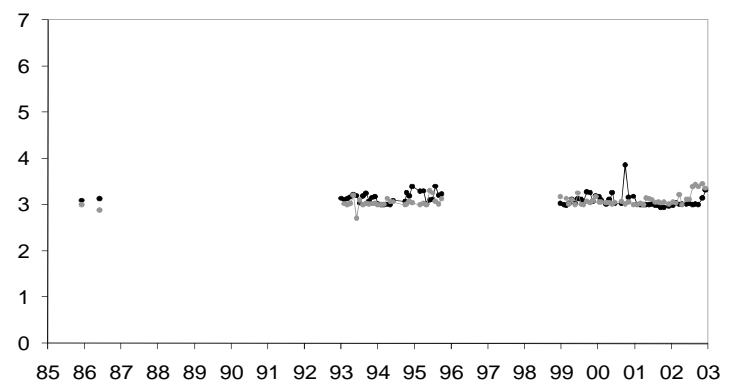
F. Shannon-Wiener



G. Berger-Parker



H. AMBI

● *Zostera noltii* site

● Bare sediment site

Figure 5

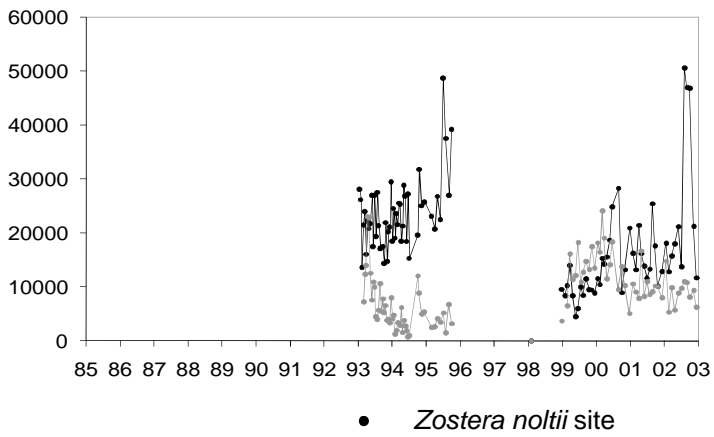
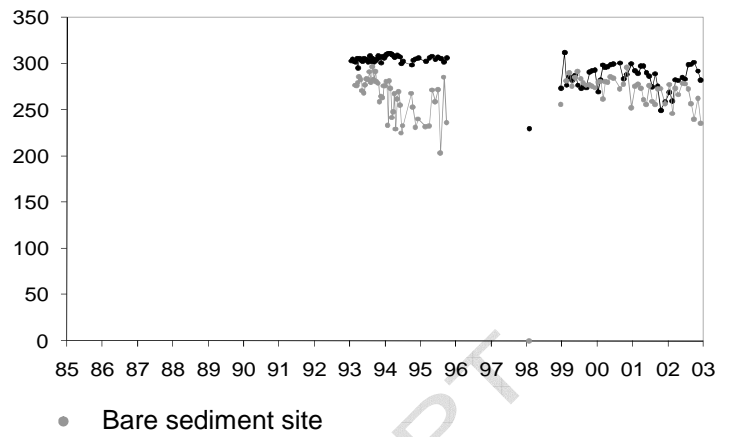
I. Eco-Exergy ($\text{g m}^{-2}\text{det.energy.equiv.}$)**J.** Specific Eco-Exergy (Eco-Exergy/biomass unit)

Figure 5 (continued)