

1 **The difficulty of disentangling natural from anthropogenic forcing factors makes the evaluation**  
2 **of ecological quality problematic: a case study from Adriatic lagoons**

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18

19 **Abstract**

20 The complex and dynamic nature of transitional ecosystems pose problems for the assessment of the  
21 Ecological Quality Status required by the European Water Framework Directive (WFD; 2000/60/EC).  
22 In six Adriatic lagoons, Ecological Quality Status was studied by comparing a biotic index based on  
23 macrophytes (MaQI), and three indices based on invertebrates (M-AMBI, M-bAMBI, and ISD).  
24 Ecological Status evaluated though MaQI and ISD resulted in quite degraded ecosystems  
25 (moderate/poor/bad), with only opportunistic algae and macrobenthic communities dominated by  
26 small size classes. Those results were supported by physico-chemical parameters, indicating high  
27 nutrients inputs, and anthropogenic pressures related with agriculture and fishery activities.  
28 Ecological Status obtained with M-AMBI and M-bAMBI was higher, with some sites reaching even  
29 the “good” status. The best response to anthropogenic pressures, in terms of a pressure index, was  
30 obtained by M-AMBI and M-bAMBI. Nevertheless, the response of used metrics (such as AMBI and  
31 bAMBI) to environmental variables not related to anthropogenic impact, and the high heterogeneity  
32 of physical—chemical conditions within lagoons, represent potential problems for the correct  
33 evaluation of Ecological Status of transitional waters. When different metrics give different responses  
34 it becomes a problem for managers who cannot easily make a decision on the remedial measures. The  
35 disagreement among indices arose because of the different response of biological elements to  
36 different stressors, and because the different indices based on macroinvertebrates focused on different  
37 aspects of the community, providing complementary information. So urge the need to find alternative  
38 approaches for a correct assessment of Ecological Status, with the combination of different biological  
39 elements, and considering the development of new indices (e.g. M-bAMBI) or refinement of the  
40 existing ones.

41

## 42 1. Introduction

43 The Water Framework Directive (WFD; 2000/60/EC) establishes a framework for the protection of  
44 European waters, including transitional waters (estuaries and lagoons). This directive invited the  
45 scientific community to undertake specific studies for the assessment of the Ecological Quality Status  
46 (EcoQ) of transitional waters (TWs). The legislation requires the quality to be defined in an  
47 integrative way using several biological elements, supported by hydro-morphological and physico-  
48 chemical quality parameters (Prato et al., 2014). In Italy, the WFD was implemented through the  
49 National Act 260/10, which indicates official methods for EcoQ assessment in TWs only for  
50 “macrophytes” (MaQI index: Sfriso et al., 2009; 2014a), and “macrozoobenthos” (M-AMBI index,  
51 Muxika et al., 2007; BITS index: Mistri and Munari, 2008). Phytoplankton and fish fauna were also  
52 indicated by the WFD as biological quality elements (BQEs) but methods to assess EcoQ have not  
53 been finalized yet, especially for fish fauna (Prato et al., 2014). For the phytoplankton the MPI -  
54 Multimetric Phytoplankton Index, is proposed by the Mediterranean Geographical Intercalibration  
55 Group (Mediterranean GIG), to assess the quality of transitional waters according to the composition  
56 of phytoplankton populations (Facca et al., 2014)

57  
58 Despite the plethora of methods to assess the EcoQ developed to date in Europe, studies on the  
59 response of assessment indices to human pressures are more scarce, and limited to few types of  
60 pressures, such as eutrophication, and other sources of pollution (Borja et al., 2013; Hering et al.,  
61 2013). As results of a recent project (WISER project, Borja et al., 2013; Hering et al., 2013) authors  
62 evaluated the need to develop new assessment methods, taking into account the lack of methods for  
63 some BQEs (e.g. macrophytes) and some ecotypes (e.g. hard substratum, lagoons, etc.). Particular  
64 attention should be given to TWs, where the assessment of EcoQ is challenging, due to the complex  
65 and dynamic nature of the ecosystem. TWs are the interfaces between the terrestrial and freshwater  
66 environments and the sea. It completely fit, as ecosystems, with the concept of ecotone, originally  
67 proposed to define ecosystem boundaries rather than true ecosystems (Basset et al., 2013). Coastal  
68 lagoons are subjected to natural disturbance which depends mainly on morphodynamics and on  
69 climatic factors, such as freshwater flooding and summer drought (Viaroli et al., 2008; Elliott and  
70 Whitfield, 2011). First attempts to show the fundamental properties of transitional waters included  
71 the Remane diagram (Remane, 1934), a conceptual model designed to show species diversity  
72 distribution along a salinity continuum and displays the numbers of species with different salinity  
73 tolerances (freshwater, brackish and marine) which comprise the communities across that continuum.  
74 Recent papers reviewed and adapted this model in order to suite to estuaries worldwide (Whitfield et  
75 al., 2012; Basset et al., 2013). The pressures on the biota and therefore characteristic and diversity of  
76 benthic communities, are shaped by the relative influence of the tides and river inputs, determining  
77 changes in salinity, nutrients and sediment transport and turbidity (Elliott and Whitfield, 2011). In  
78 TWs the effects of anthropogenic pressures are similar to those of natural variability, as expressed  
79 with the so called “Estuarine Quality Paradox” (Elliott and Quintino, 2007) making EcoQ assessment  
80 problematic and the inconsistencies between the responses of different indicators most pronounced  
81 (Borja et al., 2011). Even if a number of different indices have been developed (in particular based  
82 on macrobenthic invertebrates), to date few studies have evaluated the equivalency of EcoQ levels  
83 obtained through different BQEs for TWs (e.g. Borja et al., 2004; Curiel et al., 2012; García-Sánchez  
84 et al., 2012; Christia et al., 2014; Prato et al., 2014; Beiras, 2016).

85 In the present work the EcoQ of six lagoons, part of a transitional system (Po Delta, Northern Adriatic)  
86 heavily impacted by agricultural and fishery activities, have been evaluated through two BQEs  
87 (macrophytes and macroinvertebrates) required by WFD, using different indices. The aim was  
88 fourfold: (i) compare EcoQ based on the two BQEs: macrophytes and macrozoobenthos represented  
89 by MaQI and M-AMBI, respectively; (ii) compare EcoQ based on benthic invertebrates using M-  
90 AMBI index with other recently developed indices not required by national law (M-bAMBI and ISD);

91 (iii) compare the response of each method to anthropogenic pressures estimated as a pressure index  
92 (PI), and other environmental factors that could affect indices; (iv) for a better understanding of the  
93 differences among biotic indices, the response of single metrics (EGs, size classes, AMBI, bAMBI,  
94 H, H<sub>b</sub>, and S) to anthropogenic and environmental factors was checked.  
95

## 96 **2. Materials and Methods**

### 97 *2.1 Study area*

98 The Po Delta is a heterogeneous and dynamic complex of lagoons and ponds originating from the  
99 deposition of sediment transported by the Po River. It has high social-economic value arising from  
100 fishing, tourism and agriculture. The huge nutrient load entering from the river drainage basin,  
101 together with human activities related to agriculture, aquaculture and urban development within the  
102 Po Delta, have significantly affected the environmental equilibrium of the transitional area (Simeoni  
103 and Corbau, 2009).

104 The six lagoons considered in this study (Caleri, Marinetta, Vallona, Barbamarco, Canarin, and  
105 Scardovari, Figure 1) show a range of geomorphological and environmental features, depending on  
106 river inflows, seawater exchanges, and depth (Sfriso et al., 2014b). Depth can range from 0.5 to 1m  
107 or from 1.5 to 2.5m depending on the basin. Water column parameters, such as temperature and  
108 salinity depends on the season and the rate of freshwater and marine inputs.

109 Sediment parameters were analysed from 2008 to 2009. Sediment samples were collected with corers  
110 and retained for grain size and nutrient analyses (total carbon TC, total phosphorus TP, total nitrogen  
111 TN) according to the procedures reported in Sfriso and Marcomini (1996). Water column parameters  
112 (temperature T, salinity, oxygen saturation %DO, and pH) for the sampling periods (2008 to 2010)  
113 were obtained from the archive of the Regional Environmental Protection Agency of Veneto (ARPAV,  
114 2008-2010).

115 A total of 37 sites were analysed, 20 for macrobenthic invertebrates (4 in Caleri, 4 in Marinetta, 2 in  
116 Vallona, 2 in Barbamarco, 3 in Canarin, and 5 in Scardovari) and 17 for macrophytes (3 in Caleri, 2  
117 in Marinetta, 2 in Vallona, 3 in Barbamarco, 3 in Canarin, and 4 in Scardovari). Sampling sites were  
118 chosen to be representative of main hydrological conditions of each lagoon. Sampling of both  
119 macrophytes and invertebrates were performed during 2008, 2009, and 2010.

120 Pressures were scored (1: low, 2: moderate , 3: high) for each sampling station, as partial pressure,  
121 following an approach close to that proposed by Aubry and Elliott (2006) and Borja et al. (2011)  
122 based upon best professional judgment. A pressure index (PI) was calculated as the sum of partial  
123 pressures for each station (Table 1), and used as anthropogenic stressor to validate the results obtained  
124 with the different biotic indices.  
125

### 126 *2.2 Macrophyte sampling*

127 Total macrophyte cover of each sampling site was assessed by touching 20 times the soft bottom by  
128 a rake in order to detect the presence/absence of macroalgae. Results are reported as a percentage of  
129 the cover according to the monitoring protocols by ISPRA, 2011. One touch with the rake accounts  
130 for a cover of 5%, the limit considered in MaQI to discriminate areas where macrophytes are able to  
131 bloom from areas where the growth is hampered by disturbance factors (Sfriso et al., 2014b). The  
132 Rhodophyta/Chlorophyta ratio, another metric considered in MaQI, was obtained by sorting and wet  
133 weighting (precision  $\pm 1$  g) the macroalgae collected in 6 additional random samples by scraping the  
134 bottom for approximately 1 m with the rake all around the boat. Macrophyte subsamples  
135 representative of the collected biomass were preserved in 4% formaldehyde seawater and determined

136 at specific and intra-specific level by means of a stereo microscope and a light microscope. Some  
137 samples of doubtful identification were also kept fresh for molecular analyses.  
138

### 139 *2.3 Macrobenthic invertebrate sampling*

140 At each sampling station, three replicates were collected with a Van Veen grab (area: 0.027 m<sup>2</sup>;  
141 volume: 4 l). Samples were sieved ( $\varnothing = 0.5\text{mm}$  mesh) in the field and preserved by using a buffered  
142 solution of formaldehyde (8% in brackish water). Specimens were identified to the highest possible  
143 taxonomic separation (usually species), and their abundance was quantified as the number of  
144 individuals per sample. Animals were dried at 80°C for 48 hours in a hoven, and then incinerated at  
145 450 °C for 4 h in a muffle furnace. Each individual was weighted and the average weight per species  
146 was determined. Ash-free dried weight was considered as estimate of biomass.  
147

### 148 *2.4 Biotic indices*

149 The Ecological Status (ES) of each lagoon of the studied transitional system was assessed by applying  
150 one index based on macrophytes (MaQI), and three indices based on invertebrates (M-AMBI, M-  
151 bAMBI, and ISD).

152 Macrophyte Quality Index (MaQI), was developed and validated by Sfriso et al. (2009) and  
153 intercalibrated in the framework of the Mediterranean Geographic Intercalibration Group (Med-GIG;  
154 European Commission, 2010). After determination, each macroalgal taxon, was associated to a score  
155 according to the degree of sensitivity (0 = tolerant taxa, 1 = indifferent taxa, 2 = sensitive taxa). For  
156 the EcoQ calculation the following metrics are used: total number of species, number and percentage  
157 of sensitive macroalgal taxa, total percentage of macroalgal cover, Rhodophyta/Chlorophyta biomass  
158 ratio and percentage of aquatic angiosperm cover. The EcoQ calculation is obtained by two entries,  
159 one for macroalgae and the other for angiosperms, if present, following Sfriso et al. (2016). MaQI is  
160 a categorical index in order to be applied also in the presence of a very low cover or a few taxa, which  
161 is not possible with continuous indices. The sampling sites were classified according the following  
162 scheme: “High” if EQR = 1 or 0.85, “Good” if EQR = 0.75 or 0.65, “Moderate” if EQR = 0.55 or  
163 0.45, “Poor” if EQR = 0.35 or 0.25, and “Bad” if EQR = 0.15 or 0 (Sfriso et al., 2014).

164 Abundance (M-AMBI) and biomass (M-bAMBI) based indices were calculated using AMBI 5.0  
165 software (freely available at <http://ambi.azti.es>). For both indices invertebrates are assigned a score  
166 for I to V, based on their tolerance, following to AMBI library (Borja and Muxika, 2005). M-AMBI  
167 index is based on the following metrics: AMBI, Shannon diversity ( $H_{\log 2}$ ), and taxa richness (S)  
168 (Muxika et al., 2007). For M-bAMBI the same metrics are calculated using biomass data (Mistri et  
169 al., 2018). Reference conditions were those reported by the Italian Act 260/10, for microtidal  
170 oligo/meso/polyhaline lagoons (AMBI = 2.14;  $H' = 3.4$ ; S = 28). The sampling sites were classified  
171 according to boundaries required by Italian law for M-AMBI: “High” if  $> 0.96$ , “Good” if  $0.71 < \text{M-AMBI} \leq 0.96$ ,  
172 “Moderate” if  $0.57 < \text{M-AMBI} \leq 0.71$ , “Poor” if  $0.46 < \text{M-AMBI} \leq 0.57$ , and “Bad” if  
173  $\text{M-AMBI} \leq 0.46$ , and following Mistri et al. (2018) for M-bAMBI: “High” if  $> 0.930$ , “Good” if  
174  $0.739 < \text{M-AMBI} \leq 0.930$ , “Moderate” if  $0.632 < \text{M-AMBI} \leq 0.739$ , “Poor” if  $0.548 < \text{M-AMBI} \leq$   
175  $0.632$ , and “Bad” if  $\text{M-AMBI} \leq 0.548$ .

176 The ISD is based on the distribution of individuals across geometric size classes (class I:  $>0 - <0.2$   
177 mg, class II:  $\geq 0.2 - <0.4$  mg, class III:  $\geq 0.4 - <0.8$  mg, ... class XII:  $\geq 204.8 - <409.6$  mg). The  
178 percentage of individuals per geometric size class and station was determined and the skewness (type  
179  $G_1$ , see Joanes and Gill, 1998) of the distribution was calculated for each station, representing the ISD  
180 value (Reizopoulou and Nicolaidou, 2007). The sampling sites were classified according to the  
181 classification scale provided by Reizopoulou and Nicolaidou (2007): “High” if  $-1 \leq \text{ISD} < 1$ , “Good”  
182 if  $1 \leq \text{ISD} < 2$ , “Moderate” if  $2 \leq \text{ISD} < 3$ , “Poor” if  $3 \leq \text{ISD} < 4$ , “Bad” if  $\text{ISD} \geq 4$ . Calculations were

183 performed in R version 5.1 using libraries tidyverse, xlsx and e1017 (R Development Core Team,  
184 2008, Wickham, 2017, Dragulescu and Arendt, 2018, Meyer et al., 2018).  
185 Abundance/Biomass Comparison (ABC) method (Warwick, 1986) was used to determining the level  
186 of disturbance of benthic macrofaunal communities at each station combining the two aspect:  
187 abundance and biomass. It was used as an additional confirmatory measure of the degree of  
188 disturbance affecting macroinvertebrate community. This method involves the plotting of separate k-  
189 dominance curves for species abundance and species dominance on the same graph and W statistic  
190 (ranging from -1 to +1) was used to compare the forms of these curves. When the stress is severe  
191 communities become dominated by few or one small-bodied opportunistic species, dominating the  
192 numbers but not the biomass, therefore the abundance curve lies above the biomass curve and W tend  
193 to -1. Conversely undisturbed communities are dominated by one or few large species, dominating  
194 biomass but not abundance. Thus biomass curve lies above the abundance curve and W tend to +1.  
195

## 196 *2.5 Statistical analyses*

197 Non-parametric chi-square test applied to Kruskal-Wallis (KW) ranks (Kruskal and Wallis, 1952) was  
198 used to check if the abiotic parameters, the biotic indices and the abundance-biomass pattern changed  
199 significantly between years and lagoons. When significant differences were encountered, a Wilcoxon  
200 rank sum test (W) post hoc comparison test was also carried out.

201 The response of each index to PI, was calculated separately with the non-parametric Spearman Rank-  
202 order coefficient ( $r_s$ ) (Spearman, 1907). The same coefficient ( $r_s$ ) was calculated between PI and  
203 environmental variables, to check whether anthropogenic activities affected environmental  
204 parameters. Collinearity among environmental and biotic variables were checked in order to  
205 understand whether the effect of a variable could be masked by another one.  
206

207 Redundancy Analysis (RDA) was used to show how much of the variance of the biotic indices was  
208 related to the environmental variables and PI. RDA was chosen because it explicitly models response  
209 variables as a function of explanatory variables (Zuur et al., 2007). Ordination of the data used to  
210 calculate the correlation coefficients was performed after  $\log(x + 1)$  transformation of biotic data,  
211 using 'vegan' package for R version 3.5.1 (R Development Core Team, 2008; Oksanen et al., 2008).  
212 The results was presented as a correlation biplot (Scaling 2) displaying correlations between  
213 environmental variables, and biological parameters (EGs calculated on abundance and biomass, size  
214 classes, biotic indices: M-AMBI, M-bAMBI, ISD, and MaQI, and metrics used for indices  
215 calculation: S, H, H<sub>b</sub>, AMBI, b-AMBI). Environmental variables displayed in the biplot were chosen  
216 in order to avoid collinearity and provide the best representation of data. Permutation test under  
217 reduced model was performed to check the significance of the environmental variables.  
218

## 219 **3. RESULTS**

### 220 *3.1 Physico-chemical parameters*

221 Sediments composition differed among lagoons. Barbamarco, Canarin, Scardovari and Vallona  
222 lagoons were characterized by a dominance of silt (Table 2), and high concentration of total carbon  
223 (Table 2), nitrogen (Table 2) and phosphorous (Table 2). Conversely, sediments in Caleri and  
224 Marinetta are composed mainly by sand (Table 2), with lower percentages of total carbon (Table 2),  
225 nitrogen (Table 2) and phosphorous (Table 2). Higher percentages of shells were observed in  
226 Scardovari and Vallona (Table 2). No significant differences of physico-chemical parameters (grain  
227 size, TC, TN, TP, temperature, and salinity) were observed among lagoons nor sampling year (KW,  
228  $p > 0.05$ ), with the exception of pH showing lower values in 2009 (mean 8.0) compared to 2008 (mean

229 8.2). Conversely, a certain variability in terms of grain size was observed within some lagoons (Table  
230 2), such as Caleri (% of silt and % of sand) and Scardovari (% of shells). TN showed also a marked  
231 variability, in particular within the lagoons of Barbamarco, Caleri, and Canarin (Table 2).

232 The increasing of anthropogenic pressure (PI index, Table 1) was related to an increase of silt content  
233 ( $r_s = 0.59$ ) and total carbon content in sediments ( $r_s = 0.65$ ), together with a decrease of sand ( $r_s = -$   
234  $0.62$ ). Concentration of nutrients, in particular total carbon (TC) and total phosphorus (TP), increased  
235 with increasing percentage of silt ( $r_s > 0.92$  for TC and  $r_s > 0.77$  for TP) and decreasing content of  
236 sand ( $r_s > -0.91$  for TC and  $r_s > -0.72$  for TP).

### 237 3.2 *Macrophytes*

238 The complete absence of aquatic angiosperms in all the water bodies and the almost complete absence  
239 of sensitive species (overall only single small thalli of 4 species at Caleri) indicate a severe  
240 degradation of the ecological conditions of the entire study area. The most frequent species (Table  
241 S1) were typical of polluted areas (score = 0): *Ulva rigida* C. Agardh (73% of frequency) and  
242 *Gracilaria vermiculophylla* (Ohmi) Papenfuss (69% of frequency). On average, the number of  
243 Chlorophyta exceeded that of Rhodophyta whereas the presence of Ochrophyta was negligible  
244 showing a maximum of 3 taxa in Barbamarco in 2008. The translation of the MaQI values into classes  
245 of ecological quality results in the majority of stations being classified as “poor”, and few stations  
246 classified as “bad” (Table 3). The MaQI index and consequently the overall ecological status was  
247 more or less constant throughout the six lagoons and the studied years (KW,  $p > 0.05$ , Figure 2A),  
248 with three stations showing a decrease in ecological quality by one class (from poor to bad) from  
249 2008 to 2009, and then a return to the same proportion of 2008 the following years (Figure 3).

### 250 3.3 *Macrobenthic invertebrates*

251 Macrobenthic invertebrates community was dominated by annelids with 89% of total abundances,  
252 followed by arthropods with 8% and molluscs with 3%. The most frequent and abundant species  
253 (Table S2) were the polychaetes *Streblospio shrubsolii* (Buchanan, 1890) (98% of frequency, 52% of  
254 total abundances), and *Capitella capitata* (Fabricius, 1780) (87% of frequency, 11% of abundances).  
255 Among the most frequent species there were also oligochaetes (83% of frequency), the polychaetes:  
256 *Polydora ciliata* (Johnston, 1838) (69%), *Alitta succinea* (Leuckart, 1847) (67%), and *Spio decorata*  
257 Bobretzky, 1870 (56%), and the amphipods: *Monocorophium insidiosum* (Crawford, 1937) (58%),  
258 and *Gammarus aequicauda* (Martynov, 1931) (56%).

259 The majority of stations showed a strongly left-skewed distribution of size classes, i.e. large  
260 individuals were under-represented in the assemblages. The subsequent translation of the ISD values  
261 into classes of ecological quality results in most stations being classified as “poor”, some as  
262 “moderate”, only in 2009, two stations (Can1 and Ma2) achieved “good” status (Table 3). The  
263 calculation of M-AMBI showed overall higher ecological quality and higher variability among  
264 stations (Table 3). The majority of stations were classified as “moderate”, “poor”, and “bad”, but with  
265 some stations achieving “good” and one (Bar1) even “high” ecological status in 2010 (Table 3). The  
266 calculation of M-bAMBI showed overall high variability among stations and even higher ecology  
267 quality compared to M-AMBI, with a total of 20 station classified as “good” (Table 3).

268 The three indices analyzed (ISD, M-AMBI and M-bAMBI) showed no significant differences among  
269 the six lagoons (KW,  $p > 0.05$ ). ISD index showed differences (KW,  $p < 0.05$ ) from 2008 to 2009  
270 and from 2009 to 2010 (Figure 2B). The overall ecological status was slightly better in 2009 compared  
271 to the previous and following year, where seven stations saw an increase in ecological quality by one  
272 class (i.e. from poor to moderate or from moderate to good) from 2008 to 2009; however, in 2010 the

273 ecological status of most stations returned to the conditions of 2008 (with the exception of station  
274 Can2 which decreased in quality and station Sca3 which in 2010 achieved a moderate status, having  
275 been classified as “poor” in both 2008 and 2009). M-AMBI index (Figure 2C) and M-bAMBI index  
276 (Figure 2D) did not show significant differences among years (KW,  $p > 0.05$ ). According to M-AMBI  
277 index the ecological status of one station decreased by one class from 2008 to 2009, and signs of  
278 improvement from 2009 to 2010, with one station improving ecological status by one class (Table 3).  
279 According to M-bAMBI index four stations increased ecological quality by one class (from moderate  
280 to good) from 2008 to 2009, but in 2010 the ecological status of most stations returned to the  
281 conditions of 2008 (Table 3).

282 Overall, ISD index classified every lagoon as “poor” or “moderate” (Figure 3). Such a classification  
283 was consistent with results of M-AMBI in 2008 and 2009, but in 2010 M-AMBI classified two  
284 lagoons (Vallona and Barbamarco) as “good” (Figure 3). M-bAMBI classified Scardovari lagoon as  
285 “good” in 2008 and 2009, and “poor” in 2010, whereas indicated an improvement of environmental  
286 conditions for Caleri, Marinetta and Vallona lagoon from “poor”/“moderate” status in 2008 and 2009  
287 to “good” status in 2010 (Figure 3).

### 288 3.4 Indices validation and relation indices/pressures

289 Macrobenthic invertebrate communities were mainly represented by tolerant species (EGIII),  
290 dominating both in terms of abundances, with values ranging from 69.9% in 2008 to 77.3% in 2010  
291 (Figure 4A) mainly due to polychaetes such as *Streblospio shrubsolii*, *Hydroides dianthus* (Verrill,  
292 1873) and *Ficopomatus enigmaticus* (Fauvel, 1923), both in terms of biomass, with values ranging  
293 from 58.1% in 2008 to 61.4% in 2010 (**Errore. L'origine riferimento non è stata trovata.B**), mainly  
294 due to the bivalves *Ruditapes philippinarum* (Adams & Reeve, 1850) and *Arcuatula senhousia*  
295 (Benson, 1842). First order opportunistic species (EGV), mainly represented by the polychaete  
296 *Capitella capitata*, followed in terms of abundances (19%-8.8%), with lower percentages of sensitive  
297 species (EGI, 7%-4.9%), such as the amphipod *Gammarus aequicauda*, and second order  
298 opportunistic species (EGIV, 4.9%-4%), such as the polychaete *Polydora ciliata* and larvae of the  
299 insect *Chironomus salinarius* Kieffer, 1915. Conversely, in terms of biomass tolerant species were  
300 followed by EGI (19.3%-23.8%), mainly represented by the bivalve *Chamelea gallina* (Linnaeus,  
301 1758) and the amphipod *Gammarus aequicauda*, and EGII (14.7%-7.3%), dominated by species  
302 belonging to Actinaria, with lower percentages of EGIV (3.9%-1%), mainly represented by the  
303 bivalve *Anadara transversa* (Say, 1822) and EGV (3.5%-1%), mainly represented by the polychaete  
304 *Capitella capitata*.

305 Communities were dominated by the smallest size class (I), ranging from 78.9% of individuals in  
306 2010, to 21.5% in 2008 and 2009. The two biggest size classes (XI and XII) were not represented  
307 (Figure 4C).

308 AMBI index varied significantly from 2008 to 2010 and from 2009 to 2010 (KW and W,  $p < 0.05$ ),  
309 but differences among lagoons were at significant level (KW,  $p = 0.05$ ), whereas H and S did not  
310 showed any significant difference (KW,  $p > 0.05$ ). Neither bAMBI, nor diversity calculated on  
311 biomass ( $H_b$ ) varied significantly among years, or lagoons (KW,  $p > 0.05$ )

312 Abundance/Biomass Comparison (ABC) method (**Errore. L'origine riferimento non è stata**  
313 **trovata.D**) showed that communities were subjected to variable levels of stress, with values ranging  
314 from  $W = -0.342$ , indicating more disturbed communities, where the dominant taxa dominated for  
315 abundances, to  $W = 0.307$  indicating less disturbed communities, where the dominant taxa dominated  
316 for biomass. No significant differences were observed among years (KW,  $p > 0.05$ ), but differences  
317 were observed among lagoons (KW,  $p < 0.05$ ), in particular Canarin lagoon, showed on average  
318 higher W values, indicative of less disturbed communities, compared with Caleri, Marinetta, and  
319 Vallona. The variability within lagoons was high, as well.

320



321 Redundancy analysis did not show a clear response of biotic parameters to environmental factors  
322 (Figure 5). The variability of ecological groups (in terms of both abundance and biomass, Figure 5A),  
323 and size classes (Figure 5B) was explained most by variation of salinity (permutation test,  $p < 0.05$ ).  
324 The graphs showed a gradient of increasing salinity from right to left. Variation of biotic indices  
325 instead (Figure 5C) were explained by both salinity and PI (permutation test,  $p < 0.05$ ). The graph  
326 showed from the left to the right an increasing pattern of salinity, corresponding to increasing values  
327 of H. From the top right to the bottom left instead there was a decreasing gradient of PI, corresponding  
328 to a decreasing ISD, and increasing H<sub>b</sub>, S and M-bAMBI.

329 Comparing the different indices based on macrobenthic invertebrates M-AMBI was strongly  
330 correlated with H and S, and moderately with M-bAMBI and H<sub>b</sub>; ISD was weakly correlated with  
331 M-AMBI, bAMBI and H (Table 4)

332 Table 2. Means ( $\pm$ SD) of sediments and water parameters for each of the six studied lagoons. Water  
 333 data were obtained from ARPAV archive. TC = total carbon, TP = total phosphorus, TN = total  
 334 nitrogen), T = temperature, DO = oxygen saturation.

	<b>Barbamarco</b>	<b>Caleri</b>	<b>Canarin</b>	<b>Marinetta</b>	<b>Scardovari</b>	<b>Vallona</b>
<b>Silt (%)</b>	88.4 $\pm$ 0.8	44.2 $\pm$ 21.3	85.2 $\pm$ 11.9	21.2 $\pm$ 5.7	61.0 $\pm$ 14.2	60.6 $\pm$ 3.2
<b>Sand (%)</b>	10.3 $\pm$ 0.8	55.4 $\pm$ 20.8	14.1 $\pm$ 11.8	78.4 $\pm$ 5.9	33.9 $\pm$ 12.8	37.1 $\pm$ 1.6
<b>Shells (%)</b>	1.3 $\pm$ 0.0	0.4 $\pm$ 0.6	0.7 $\pm$ 0.2	0.4 $\pm$ 0.2	5.1 $\pm$ 1.4	2.3 $\pm$ 1.7
<b>TC (mg/g)</b>	35.5 $\pm$ 2.7	27.3 $\pm$ 6.7	34.7 $\pm$ 0.2	24.8 $\pm$ 0.2	31.0 $\pm$ 0.4	31.8 $\pm$ 1.5
<b>TN (mg/g)</b>	1.8 $\pm$ 0.6	1.0 $\pm$ 0.7	2.1 $\pm$ 0.8	0.7 $\pm$ 0.0	1.7 $\pm$ 0.3	1.3 $\pm$ 0.0
<b>TP (<math>\mu</math>g/g)</b>	647.4 $\pm$ 24.4	566.9 $\pm$ 70.4	633.1 $\pm$ 40.6	513.3 $\pm$ 22.9	544.0 $\pm$ 16.2	561.2 $\pm$ 64.8
<b>Temp (<math>^{\circ}</math>C)</b>	17.8 $\pm$ 0.7	18.9 $\pm$ 1.1	18.5 $\pm$ 0.3	18.2 $\pm$ 1.9	18.8 $\pm$ 1.4	18.0 $\pm$ 2.0
<b>Salinity</b>	25.6 $\pm$ 2.8	26.9 $\pm$ 2.7	22.8 $\pm$ 0.3	22.8 $\pm$ 1.6	26.8 $\pm$ 1.9	21.2 $\pm$ 0.3
<b>DO (%)</b>	99.8 $\pm$ 3.4	112.3 $\pm$ 2.5	109.7 $\pm$ 15.6	103.2 $\pm$ 3.0	108.3 $\pm$ 8.8	94.7 $\pm$ 4.4
<b>pH</b>	8.3 $\pm$ 0.1	8.2 $\pm$ 0.2	8.3 $\pm$ 0.1	8.0 $\pm$ 0.2	8.2 $\pm$ 0.1	8.0 $\pm$ 0.1

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337 Table 3. Number of stations per Ecological Quality Status in each study year, using different  
 338 indices.

<b>MaQI</b>			
<b>Status</b>	<b>2008</b>	<b>2009</b>	<b>2010</b>
<b>Poor</b>	15	12	15
<b>Bad</b>	2	5	2
<b>Total</b>	17	17	17
<b>ISD</b>			
<b>Status</b>	<b>2008</b>	<b>2009</b>	<b>2010</b>
<b>Good</b>	0	2	0
<b>Moderate</b>	4	6	2
<b>Poor</b>	16	12	10
<b>Total</b>	20	20	12
<b>M-AMBI</b>			
<b>Status</b>	<b>2008</b>	<b>2009</b>	<b>2010</b>
<b>High</b>	0	0	1
<b>Good</b>	4	3	2
<b>Moderate</b>	7	8	4
<b>Poor</b>	4	4	4
<b>Bad</b>	5	5	1
<b>Total</b>	20	20	12
<b>M-bAMBI</b>			
<b>Status</b>	<b>2008</b>	<b>2009</b>	<b>2010</b>
<b>Good</b>	6	10	4
<b>Moderate</b>	6	2	3
<b>Poor</b>	3	3	1
<b>Bad</b>	5	5	4
<b>Total</b>	20	20	12

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343 Table 4). M-AMBI showed the best response to PI (Table 4), followed by Shannon index calculated  
344 on abundances (H) and biomass (H<sub>b</sub>), M-bAMBI, S and ISD. MaQI index, AMBI and bAMBI showed  
345 no significant correlation with PI (Table 4). S was also correlated with salinity (Table 4), while H  
346 was also correlated with oxygen (Table 4). AMBI values increased with increasing salinity (Table 4),  
347 while bAMBI was negatively correlated with salinity (Table 4).

#### 348 4. Discussion

349 The lagoons of the Po Delta are heavily affected by different anthropogenic pressures, mainly by high  
350 nutrient and pollutant inputs through river outflows and water turbidity due both to the erosion of the  
351 riverbanks, always deprived of vegetation, and intense fishing activities to catch the Manila clam  
352 (*Ruditapes philippinarum* Adams & Reeve) occurring in many of these areas (e.g. Munari et al., 2010;  
353 Sfriso et al., 2016; Maggi et al., 2017; Franzo and Del Negro, 2019). The degraded ecological  
354 conditions of these environments were known even without the application of indices of ecological  
355 status (Sfriso et al., 2016). The strength of those pressures varied among and within lagoons.  
356 Chemical data from the studied period did not suggest any severe dystrophic events, with dissolved  
357 oxygen values around saturation levels. Nevertheless, cases of water stratification and hypoxic  
358 conditions (oxygen concentration closed to the bottom: 1 mg/l) were reported in some basins in other  
359 periods of the year (ARPAV, 2008-2010), and those events could have had a long-term effect on  
360 macrobenthic communities. Moreover, recent investigations indicates high nutrient availability in Po  
361 Delta lagoons, with seasonal and local variations. In particular dissolved inorganic nitrogen showed  
362 maximum values (>30µM) higher than limits proposed in the National Act 260/10, and reactive  
363 phosphorous showed a maximum value of 24.9 µM, never recorded previously in any other Italian  
364 TWs (Sfriso et al., 2016). In the present work, the relation between PI and total carbon content in  
365 sediments confirmed that nutrients inputs represented one of the main anthropogenic pressures.  
366 Nevertheless, nutrient content (TC and TP) was higher where silt predominated. Sediments grain size  
367 is considered one of the indicators of the potential capacity of the system to react to pollutants: the  
368 presence of cohesive sediments maintain water even during emersion, and enable no lateral  
369 movement or percolation for water and oxygen, therefore fine sediments are more prone to anoxic  
370 condition and sulphide production (Viaroli et al., 2008). The lack of correlation between PI and other  
371 parameters, such as oxygen and TN suggests that some components of anthropogenic impacts could  
372 be not fully explained by the index PI. Moreover, sediments can also have a direct effect on benthic  
373 community, so the variability of environmental parameters (grain size and TN) observed within  
374 Barbamarco, Caleri, Canarin, and Scardovari lagoons, can act as confounding factor when comparing  
375 the effect of impacts. Salinity is also highly variables within each transitional water body, as result of  
376 the combined effects of hydromorphology, river and marine influence (Elliott and Whitfield, 2011,  
377 Whitfield et al., 2012; Basset et al., 2013). Other authors have pointed out that a lagoon should not  
378 be considered spatially uniform and unique unit but as a mosaic of assemblages when applying the  
379 EU Water Framework Directive or assessing environmental impact (Pérez-Ruzafa et al., 2008).  
380 Sampling design could help controlling the effect of natural variability, but temporal, together with  
381 spatial pattern should be taken into account (Khedhri et al., 2017; Pasqualini et al., 2017). In the  
382 present work it was not possible to detect a clear spatial or temporal pattern of abiotic parameters  
383 within each lagoon due to the patchiness of environmental conditions and the complexity of the  
384 relationship between temporal and spatial changes. Our results are in line with an investigation  
385 performed within a hypersaline coastal lagoon in the south-western Mediterranean, where  
386 macrophyte assemblages diversity and richness responded more to the frequency, regularity and  
387 intensity of environmental fluctuations than salinity or confinement gradients themselves (Pérez-  
388 Ruzafa et al., 2008). The numerous natural and anthropogenic stressors in TWs provoke a high level

389 of habitat fragmentation, forcing species and communities to adapt to such heterogeneous conditions  
390 (Prato et al., 2014).

391 In general all indicators used confirmed the general degradation of Po Delta lagoons, with most  
392 sample assigned to a EcoQ below the critical Good/Moderate threshold, but in some cases the use of  
393 different BQEs and different indices based on the same BQE lead to different EcoQ. Moreover,  
394 different biotic indices showed differential response to both environmental parameters and  
395 anthropogenic pressures.

396 Macrophyte composition and MaQI index values lead to classify the ES of all Po Delta lagoons, as  
397 “poor” or “bad”. Even if in the past the presence of *Ruppia cirrhosa* (Petagna) Grande was reported  
398 for Po Delta system (Sfriso et al., 2016), angiosperms have totally disappeared, and were never  
399 recorded in the studied period. The presence of very few sensitive algal species, and the dominance  
400 of free-floating opportunistic macroalgae (e.g. genera *Ulva* and *Gracilaria*) is a symptom of  
401 eutrophication and degradation of the environment (Viaroli et al., 2008). One of the major driver of  
402 the shifts from the dominance of angiosperms and sensitive macroalgal species to blooms of  
403 opportunistic and nitrophilous macroalgae is the increase of nutrient loads, in particular nitrogen  
404 (Viaroli et al., 2008; Sfriso et al., 2016). Some stations were evaluated as “bad”, because of the  
405 extremely low macroalgal cover (<5%) or their total absence. Such a condition indicates severe  
406 degradation: when waters are so turbid that light cannot penetrate to the bottom, macroalgae slow  
407 down their growth and phytoplankton and cyanobacteria become the only primary producers (Viaroli  
408 et al., 2008; Sfriso et al., 2016). Even if this transitional system is part of the Regional Po Delta Park,  
409 the area is completely surrounded by cultivated fields, affecting the transitional system with the high  
410 nutrient loads, and likely also with chemicals and other toxic substances. For instance, sediments  
411 analyzed in 2008, showed Hg concentration higher than limits established by WFD (> 0.3 mg/kg),  
412 with concentration up to three times higher than this threshold in one station in Vallona lagoon  
413 (*unpublished results*). Investigation at lower levels of biological organization, with tools such as  
414 ecotoxicological biomarkers, could provide more detailed information on the biotic response to this  
415 type of stressors (Beiras, 2016). Fishery activities could also be detrimental for macrophytes,  
416 destroying the natural sediment texture and resuspending high amounts of fine sediments which  
417 dramatically reduce light availability favoring cylindrical and filamentous thalli (Sfriso et al., 2016).  
418 The *Poor/Bad* ES evaluated with MaQI enhanced the necessity of a policy of interventions to improve  
419 conditions and achieve the *Good* ecological status as requested by the WFD. Those results were  
420 consistent with the high level of anthropogenic pressures affecting the studied area, even if the  
421 extremely reduced differences of ES between samples lead to no significant correlation between  
422 MaQI index and PI, nor between MaQI and other environmental variables.

423 The indices based on macrobenthic invertebrates in general gave higher scores compared with  
424 evaluation based on macrophytes. The disagreement of the EcoQ assessment obtained through  
425 different BQEs is particularly marked in TWs (Borja et al., 2011) and arose because of the different  
426 response of biological elements to different stressors. Macroalgae were considered more sensitive to  
427 eutrophication, seagrasses to hydromorphological changes or habitat loss (Borja et al., 2013), and  
428 benthic invertebrates to “general degradation” (Hering et al., 2013). ISD index represents the  
429 skewness of the distribution of individuals of a benthic community in geometric size (biomass)  
430 classes and is an alternative method to investigate benthic community structure (Reizopoulou and  
431 Nicolaidou, 2007). Body size abundance distribution is suggested to be related to disturbance pressure  
432 through individual energetics, population dynamics, interspecific interactions and species coexistence  
433 responses. Even if ISD index did not show the best response to PI, the EcoQ obtained was the most  
434 consistent with EcoQ based on macrophytes (MaQI): all analyzed lagoons never reached a *Good*  
435 environmental status. In general ISD index gave, with few exceptions, lower scores compared with  
436 M-AMBI and M-bAMBI, and this discrepancy was between *Moderate* and *Good* status at some sites.

437 M-AMBI and M-bAMBI indices combine two aspects of macroinvertebrate community: qualitative  
438 (sensitivity of each species: AMBI and bAMBI), and quantitative (structural indices: H, H<sub>b</sub>, and S).  
439 In the present work M-AMBI and M-bAMBI, showed the best response to PI, but they showed a  
440 discrepancy with the other two indices, which was more marked for M-bAMBI (higher number of  
441 samples classified as *Good*). Since this discrepancy crossed the critical boundary between *Moderate*  
442 and *Good* status, the results of those indices should be considered with caution: assessing as *Good* a  
443 site which is actually *Moderate* could result in underestimating the necessity of management actions  
444 and vice versa.

445 In general, with few exceptions (Canarin in 2008-2010, and Barbamarco in 2010), ES calculated with  
446 M-bAMBI corresponded or was higher than EcoQ calculated with M-AMBI. This was the result of  
447 the highest percentages represented by sensitive and indifferent species when biomass was  
448 considered, and it's a direct consequence of the biological traits of r- and k-strategists (Pearson and  
449 Rosenberg, 1978). All biotic indices considered in the present work are based on the paradigm stating  
450 that the increasing organic pollution results in loss of the larger long-lived species (k-strategists) from  
451 the community in favor of more tolerant short-lived opportunists (r-strategists) (Pearson and  
452 Rosenberg, 1978). Nevertheless, the different metrics used to quantify the alternative states of this  
453 phase shift provided different results. While M-AMBI (and AMBI) quantify the effect of organic  
454 pollution only in terms of species abundances, ISD, and M-bAMBI (and bAMBI) considered also  
455 that k-strategists dominate in terms of biomass, while r-strategists in terms of abundance  
456 (Reizopoulou and Nicolaidou, 2007). The theory of changes of benthic community biomass under  
457 disturbed conditions, well documented in benthic ecology (Pearson and Rosenberg, 1978), was also  
458 at the base of ABC analysis. In the present work, differently from biotic indices ABC method did not  
459 discriminate among years, but among lagoons, with also a high within-lagoon variability, suggesting  
460 it to be more sensible to local changes, representing the sum of particular conditions, most of them  
461 limited in space. It was already pointed out that ABC methods proved to be efficient in defining  
462 whether the community was subjected to stress, but it could be biased by recruitment (Beukema,  
463 1988) and it does not always discriminate between natural and anthropogenic causes of such a stress  
464 (Clarke and Warwick, 2001; Lardicci et al., 2001). This factor is crucial in TWs, where organisms  
465 have to cope with the high natural variability of environmental parameters, resulting in the dominance  
466 of tolerant species (EGIII), in terms of both abundances and biomass, a common feature of such  
467 environments (e.g. Marchini et al., 2008; Pitacco et al., 2018). Such species did not show any  
468 correlation with PI nor environmental variables, but given their dominance, they have a critical role  
469 in the assessment of ES, posing problems to the applicability of those biotic indices in TWs.  
470 Moreover, the pattern of sensitive and opportunistic species was not consistent with PI and  
471 environmental variables, as well. This lack of response could be related to a possible adaptation of  
472 local species living in TWs to stressed conditions. Most species living in TWs adapt to such variations  
473 (Cognetti, 1992) and become tolerant of changes (Cognetti and Maltagliati, 2000). In fact, analyses  
474 on genetic divergence on brackish species showed a high degree of fragmentation in local population,  
475 morphologically unidentifiable, with different degrees of adaptability (Cognetti and Maltagliati,  
476 2000). Other authors have pointed out the need to revise the concept of r/K selection concept in TWs.  
477 Pérez-Ruzafa et al. (2013) found that estuarine fish species combine r and K characteristics,  
478 suggesting lagoonal selection would not necessarily act on all the biological traits of a species but  
479 only on some of them, improving the adaptation of local populations to the lagoon environment but  
480 upsetting the coherence of all biological traits in an r/K context. Previously Stearns (1977) had  
481 observed that in fish assemblages r and K strategists are not necessarily negatively correlated.

482 The WFD (2000/60/EC) requires that any method used to assess the ecological status must detect  
483 only anthropogenic pressures, show a clear pressure-response, and avoid the detection of natural  
484 variability (Reiss and Kröncke, 2005). In TWs, however, stress of both natural and anthropogenic

485 origin create a variety of conditions that make it difficult to disentangle the effect of anthropogenic  
486 activities from naturally induced stress (the so-called “Estuarine Quality Paradox”; Elliott and  
487 Quintino, 2007; Dauvin, 2007). In the present work biotic indices based on macrobenthic  
488 invertebrates showed significant relationships with anthropogenic disturbances (in terms of PI), and  
489 seemed robust to natural variability. Some of the metrics used (AMBi, bAMBI and S) for calculation  
490 of biotic indices were also related with PI, but also with variation of environmental variables, in terms  
491 of salinity and oxygen. Oxygen is one of the elements to be monitored according to National Act  
492 260/10, since its low concentration is an indication of a dystrophic crises, and therefore degraded  
493 conditions. Conversely, salinity is related with natural hydromorphological characteristics of the  
494 lagoons, not with anthropogenic pressures, and moreover is subjected to daily and seasonal variations.  
495 Therefore, this relationship represented a potential problem for the correct evaluation of ES.

496 The metrics of indices based on macroinvertebrates resulted the most affected by natural variability.  
497 In particular the species richness and Shannon–Wiener Index calculated on abundances (H) was the  
498 metric showing the highest correlation with environmental parameters. The unreliability of univariate  
499 indices for a correct evaluation of EcoQ was already pointed out by other authors. Those indices  
500 showed high variability related to seasonality, making than less suitable for the aim of WFD (Reiss  
501 and Kröncke, 2005). Indeed, diversity showed also a strong negative correlation with confinement  
502 (Reizopoulou and Nicolaidou, 2004), which is a natural situation not always associated with  
503 environmental health. Biotic indices such as AMBI resulted more stable with respect to seasonality  
504 (Reiss and Kröncke, 2005), but in the present work they responded better to salinity gradient than to  
505 PI, probably due to the fact that the percentage of ecological groups did not respond in coherent way  
506 to PI as well. The effect of salinity on biotic indices is well known, and in the National Act 260/10  
507 transitional water bodies are classified on the basis of tide and salinity, with the aim of reducing the  
508 bias. Nevertheless, the present work showed that the high variability of salinity in transitional waters  
509 can influence indices also within the same water typology (the whole Po Delta system fall in the  
510 category of “microtidal oligo/poly/mesohaline). M-AMBI and M-bAMBI indices combine two  
511 aspects of macroinvertebrate community: qualitative (sensitivity of each species: AMBI and bAMBI),  
512 and quantitative (structural indices: H, H<sub>b</sub>, and S), and they showed the best response to PI, without  
513 bias related to salinity.

514 The richness of macrophytes at inter-lagoonal level are also known to be influenced by salinity (e.g.  
515 Pérez-Ruzafa et al., 2011; Schubert et al., 2011; Janousek and Folger, 2012) generally showing higher  
516 values with higher salinity. Nevertheless, in pristine conditions the few species present have high  
517 ecological value, such as angiosperms (Sfriso et al., 2016, and references therein), and this makes the  
518 macrophytes a more stable BQE in eutrophic areas with high salinity variations, such as transitional  
519 areas. Our results highlighted the efficiency of combining different metrics and the necessity of  
520 correcting the metrics for salinity and other confounding environmental variables (see for instance  
521 Leonardsson et al., 2016).

522 The metrics used to assess the EcoQ must be able to discriminate between natural and anthropogenic  
523 changes otherwise false conclusions may be reached regarding the status being as the result of human  
524 pressures. In this view further efforts are still needed to implement the efficiency of EcoQ assessment  
525 methods in TWs. A correct classification of transitional ecosystems into discrete categories of  
526 ecological status can be best achieved by combining different BQEs, and refining biotic indices in  
527 order to improve their efficiency in TWs. The WFD (2000/60/EC) uses the “one-out, all-out”  
528 principle to combine assessments from different BQEs. This principle is based upon the assumption  
529 that the worst status of the elements used in the assessment determines the final status of a water body  
530 (Borja et al., 2004). This method was efficient in determining the ES in the highly impacted systems  
531 objected of the present study, but tend to inflate type I error, and resulted in an underestimation of  
532 EcoQ (below *Good* status, when it was actually *Good*) in less impacted transitional systems (Hering

533 et al., 2013; Prato et al., 2014). This could become a critical step, in particular when conditions are at  
534 the border between *Moderate/Good* status. Further works analyzing the uncertainty of defining  
535 boundaries and calculating the confidence of ecological classes, with methods already used within  
536 the WFD framework for a UK freshwater macrobenthic dataset (Clarke, 2013), could improve our  
537 understanding on those critical boundaries. A further difficulty in disentangling natural and  
538 anthropogenic stressors is due to the lack of standard and objective methods to estimate anthropogenic  
539 impacts. The quantitative assessment of anthropogenic pressures, commonly expressed as a pressure  
540 index (PI), required expert judgment and could be biased by a certain level of subjectivity. In the  
541 present work, both biotic and abiotic data clearly support the accuracy of such quantification, but the  
542 homogenization of EcoQ in the study system limited the usefulness of the correlation with PI to assess  
543 the efficiency of biotic indices. The difficulties inherent to the complex and dynamic nature of  
544 transitional ecosystems urge the need to find alternative approaches for a correct EcoQ assessment.  
545 In this view the recently developed biomass-based indices seem promising, providing complementary  
546 ecologically relevant information with respect to previous ones, downscaling the effects of over  
547 abundant small-bodied organisms (Mistri et al., 2018).

548

## 549 **5. Summary**

550 The lagoons of the Po Delta are heavily affected by different anthropogenic pressures, mainly by high  
551 nutrient and pollutant inputs through river outflows, water turbidity and intense agricultural and  
552 fishing activities. The strength of those pressures varied among and within lagoons. It was not  
553 possible to detect a clear spatial or temporal pattern of abiotic parameters within each lagoon due to  
554 the patchiness of environmental conditions and the complexity of the relationship between temporal  
555 and spatial changes. In general the indicators used confirmed the general degradation of Po Delta  
556 lagoons, with most sample assigned to a EcoQ below the critical *Good/Moderate* threshold, but in  
557 some cases the use of different BQEs and different indices based on the same BQE lead to different  
558 EcoQ.

559 Macrophyte composition and MaQI index values lead to classify the ES of all Po Delta lagoons, as  
560 *Poor* or *Bad*. The indices based on macrobenthic invertebrates in general gave higher scores  
561 compared with evaluation based on macrophytes. The disagreement arose because of the different  
562 response of biological elements to different stressors. In general ISD index gave lower scores  
563 compared with M-AMBI and M-bAMBI, and this discrepancy was critical at some sites because it  
564 was between *Moderate* and *Good* status. Discrepancies were more marked between ISD and M-  
565 bAMBI, and arose because they focused on different aspects of the community, providing therefore  
566 complementary information.

567 MaQI results were consistent with the high level of anthropogenic pressures affecting the studied  
568 area. Nevertheless, the extremely reduced differences of ES between samples lead to no significant  
569 correlation between MaQI index and PI, nor between MaQI and other environmental variables,  
570 suggesting it was not sensitive to minor differences among lagoons. Conversely, biotic indices based  
571 on macrobenthic invertebrates were significantly correlated with anthropogenic disturbance  
572 expressed in terms of PI, suggesting this BQE is more sensitive to changes also in highly degraded  
573 condition. Nevertheless, the discrepancy among indices between the critical boundary  
574 *Good/Moderate*, indicate caution: assessing as *Moderate* a site which is actually *Good* could result  
575 in unnecessary management actions and vice versa.

576 Some of the metrics used for calculation of biotic indices were also related with PI, but also with  
577 variation of environmental variables, in terms of oxygen and salinity. Salinity is related with natural



578 hydromorphological characteristics of the lagoons, not with anthropogenic pressures. This  
579 relationship, together with the infra-lagoon variability, represented a potential problem for the correct  
580 evaluation of ES and highlight the need of metrics correction for possible confounding environmental  
581 parameters. A combination of different BQEs and different indices, with a refinement of some  
582 existing indices, would be therefore crucial to improve EcoQ classification.

583

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586

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754

756 Table 1. Pressures at the various sampling sites. Modified from Mistri et al., 2018.

	Site	Non-point pollution sources		Point pollution sources			Habitat loss		Ports			Fisheries		PI
		Agricultural inputs	Freshwater inputs	Domestic	Agricultural	Industrial	Land-claim	Physical alteration	Port activity	Navigation	Dredging	Fin-fisheries	Shell-fisheries	
<b>Caleri</b>	Cal1	2			2							1	3	<b>8</b>
	Cal2	2			2							1	1	<b>6</b>
	400	2			2							1	1	<b>6</b>
	Cal3	1			1								2	<b>4</b>
	220	1			1								2	<b>4</b>
	Cal4	1		1								1	1	<b>4</b>
	210	1		1								1	1	<b>4</b>
<b>Marinetta</b>	Ma1	1											2	<b>3</b>
	Ma2	1											2	<b>3</b>
	410	1											2	<b>3</b>
	Ma3	1	3		2								2	<b>8</b>
	230	1	3		2								2	<b>8</b>
	Ma4	1						2					2	<b>5</b>
<b>Vallona</b>	Val1	1	1		1			1					3	<b>7</b>
	Val2	1	1		1								3	<b>6</b>
	250	1	1		1								3	<b>6</b>
	240	1											2	<b>3</b>
<b>Barbamarco</b>	420	2	1		2		1						3	<b>9</b>
	Bar1	2			2		1						3	<b>8</b>
	270	2			2		1	2					2	<b>9</b>
	Bar2	2			2		1						3	<b>8</b>
	260	2			2		1						3	<b>8</b>
<b>Canarin</b>	Can1	1											2	<b>3</b>
	430	1											2	<b>3</b>
	Can2	1			2						1	3	<b>7</b>	
	440	1			2						1	3	<b>7</b>	
	Can3	1	1		2						1	3	<b>8</b>	
	290	1	1		2						1	3	<b>8</b>	
<b>Scardovari</b>	Sca1	1					1						2	<b>4</b>
	330	1					1						2	<b>4</b>
	Sca2	1											3	<b>4</b>
	Sca3	1											2	<b>3</b>
	340	1	1										2	<b>4</b>
	Sca4	1	1		2						1	2	<b>7</b>	
	450	1	1		2						1	2	<b>7</b>	
	Sca5	1					1			1			1	<b>4</b>
	320	1					1			1			1	<b>4</b>

758 Table 2. Means ( $\pm$ SD) of sediments and water parameters for each of the six studied lagoons. Water  
 759 data were obtained from ARPAV archive. TC = total carbon, TP = total phosphorus, TN = total  
 760 nitrogen), T = temperature, DO = oxygen saturation.

	<b>Barbamarco</b>	<b>Caleri</b>	<b>Canarin</b>	<b>Marinetta</b>	<b>Scardovari</b>	<b>Vallona</b>
<b>Silt (%)</b>	88.4 $\pm$ 0.8	44.2 $\pm$ 21.3	85.2 $\pm$ 11.9	21.2 $\pm$ 5.7	61.0 $\pm$ 14.2	60.6 $\pm$ 3.2
<b>Sand (%)</b>	10.3 $\pm$ 0.8	55.4 $\pm$ 20.8	14.1 $\pm$ 11.8	78.4 $\pm$ 5.9	33.9 $\pm$ 12.8	37.1 $\pm$ 1.6
<b>Shells (%)</b>	1.3 $\pm$ 0.0	0.4 $\pm$ 0.6	0.7 $\pm$ 0.2	0.4 $\pm$ 0.2	5.1 $\pm$ 1.4	2.3 $\pm$ 1.7
<b>TC (mg/g)</b>	35.5 $\pm$ 2.7	27.3 $\pm$ 6.7	34.7 $\pm$ 0.2	24.8 $\pm$ 0.2	31.0 $\pm$ 0.4	31.8 $\pm$ 1.5
<b>TN (mg/g)</b>	1.8 $\pm$ 0.6	1.0 $\pm$ 0.7	2.1 $\pm$ 0.8	0.7 $\pm$ 0.0	1.7 $\pm$ 0.3	1.3 $\pm$ 0.0
<b>TP (<math>\mu</math>g/g)</b>	647.4 $\pm$ 24.4	566.9 $\pm$ 70.4	633.1 $\pm$ 40.6	513.3 $\pm$ 22.9	544.0 $\pm$ 16.2	561.2 $\pm$ 64.8
<b>Temp (<math>^{\circ}</math>C)</b>	17.8 $\pm$ 0.7	18.9 $\pm$ 1.1	18.5 $\pm$ 0.3	18.2 $\pm$ 1.9	18.8 $\pm$ 1.4	18.0 $\pm$ 2.0
<b>Salinity</b>	25.6 $\pm$ 2.8	26.9 $\pm$ 2.7	22.8 $\pm$ 0.3	22.8 $\pm$ 1.6	26.8 $\pm$ 1.9	21.2 $\pm$ 0.3
<b>DO (%)</b>	99.8 $\pm$ 3.4	112.3 $\pm$ 2.5	109.7 $\pm$ 15.6	103.2 $\pm$ 3.0	108.3 $\pm$ 8.8	94.7 $\pm$ 4.4
<b>pH</b>	8.3 $\pm$ 0.1	8.2 $\pm$ 0.2	8.3 $\pm$ 0.1	8.0 $\pm$ 0.2	8.2 $\pm$ 0.1	8.0 $\pm$ 0.1

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763 Table 3. Number of stations per Ecological Quality Status in each study year, using different  
 764 indices.

<b>MaQI</b>			
<b>Status</b>	<b>2008</b>	<b>2009</b>	<b>2010</b>
<b>Poor</b>	15	12	15
<b>Bad</b>	2	5	2
<b>Total</b>	17	17	17
<b>ISD</b>			
<b>Status</b>	<b>2008</b>	<b>2009</b>	<b>2010</b>
<b>Good</b>	0	2	0
<b>Moderate</b>	4	6	2
<b>Poor</b>	16	12	10
<b>Total</b>	20	20	12
<b>M-AMBI</b>			
<b>Status</b>	<b>2008</b>	<b>2009</b>	<b>2010</b>
<b>High</b>	0	0	1
<b>Good</b>	4	3	2
<b>Moderate</b>	7	8	4
<b>Poor</b>	4	4	4
<b>Bad</b>	5	5	1
<b>Total</b>	20	20	12
<b>M-bAMBI</b>			
<b>Status</b>	<b>2008</b>	<b>2009</b>	<b>2010</b>
<b>Good</b>	6	10	4
<b>Moderate</b>	6	2	3
<b>Poor</b>	3	3	1
<b>Bad</b>	5	5	4
<b>Total</b>	20	20	12

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769 Table 4. Spearman correlation coefficients ( $r_s$ ) between pressure index (PI), and environmental  
 770 parameters (salinity and oxygen), and indices based on macrobenthic invertebrates (MaQI, ISD, M-  
 771 AMBI, M-bAMBI), and metrics used to calculate them (AMBI, H, bAMBI,  $h_b$ , S). Only abiotic  
 772 parameters significantly correlated with biotic ones are displayed. *NS*:  $p > 0.05$ , Significant  
 773 correlations ( $p < 0.05$ ) in bold.

		Indices					Metrics			
		MaQI	ISD	M-AMBI	M-bAMBI	AMBI	H	bAMBI	$h_b$	S
Stressors	PI	<i>NS</i>	<b><math>r_s = 0.31</math></b>	<b><math>r_s = -0.48</math></b>	<b><math>r_s = -0.36</math></b>	<i>NS</i>	<b><math>r_s = -0.38</math></b>	<i>NS</i>	<b><math>r_s = -0.38</math></b>	<b><math>r_s = -0.35</math></b>
	Salinity	<i>NS</i>	<i>NS</i>	<i>NS</i>	<i>NS</i>	<b><math>r_s = -0.63</math></b>	<i>NS</i>	<b><math>r_s = -0.6</math></b>	<i>NS</i>	<b><math>r_s = 0.61</math></b>
	Oxygen	<i>NS</i>	<i>NS</i>	<i>NS</i>	<i>NS</i>	<i>NS</i>	<b><math>r_s = 0.62</math></b>	<i>NS</i>	<i>NS</i>	<i>NS</i>
Indices	ISD			<b><math>r_s = 0.28</math></b>	<i>NS</i>	<i>NS</i>	<b><math>r_s = 0.33</math></b>	<b><math>r_s = 0.29</math></b>	<i>NS</i>	<i>NS</i>
	M-AMBI				<b><math>r_s = 0.61</math></b>	<i>NS</i>	<b><math>r_s = 0.86</math></b>	<i>NS</i>	<b><math>r_s = 0.56</math></b>	<b><math>r_s = 0.71</math></b>
	M-bAMBI						<b><math>r_s = 0.50</math></b>	<i>NS</i>	<b><math>r_s = 0.76</math></b>	<b><math>r_s = 0.79</math></b>
Metrics	AMBI						<i>NS</i>	<i>NS</i>	<i>NS</i>	<i>NS</i>
	H							<i>NS</i>	<b><math>r_s = 0.39</math></b>	<b><math>r_s = 0.56</math></b>
	bAMBI								<i>NS</i>	<i>NS</i>
	$h_b$									<b><math>r_s = 0.52</math></b>

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776 Table 5. Summary of advantages and disadvantages of the use of different indices and metrics,  
 777 combining results of present work and literature.

		<b>Advantages</b>	<b>Disadvantages</b>
<b>Indices</b>	<b>MaQI</b>	effective also with extremely low macroalgal cover (<5%), robust to variation of salinity, consistent with eutrophication response to PI, and not to environmental parameters (present work)	unable to detect changes in heavily degraded conditions (present work)
	<b>ISD</b>	response to PI, and not to environmental parameters (present work)	
	<b>M-AMBI</b>	response to PI, and not to environmental parameters (present work)	critical uncertainty acrossed the <i>Moderate/Good</i> boundary
	<b>M-bAMBI</b>	response to PI, and not to environmental parameters (present work)	critical uncertainty acrossed the <i>Moderate/Good</i> boundary
<b>Metrics</b>	<b>H</b>	response to PI, and oxygen (present work)	high variability related to seasonality (Reiss and Kröncke, 2005), correlation with confinement (Reizopoulou and Nicolaidou, 2004)
	<b>H<sub>b</sub></b>	use of ecollogically relevant information (biomass) (present work, Mistri et al., 2018), response to PI (present work)	
	<b>S</b>	response to PI (present work)	response to salinity (present work)
	<b>AMBI</b>	stable with respect to seasonality (Reiss and Kröncke, 2005)	response to salinity (present work)
	<b>bAMBI</b>	use of ecollogically relevant information (biomass) (present work, Mistri et al., 2018)	response to salinity (present work)
	<b>W index</b>	sensitive to disturbance (Clarke and Warwick, 2001)	high within-lagoon variability (present work), biased by recruitment (Beugema, 1988), no discrimination between natural and anthropogenic stress (Clarke and Warwick, 2001; Lardicci et al., 2001)

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779

780 **Figure legends**

781 Figure 1. Map of the studied sites

782 Figure 2. Boxplot showing the distribution of values of MaQI (A), ISD (B), M-AMBI (C), M-bAMBI  
783 (D) in each study year. Midline = median; upper limits of the box = third quartile (75th percentile);  
784 lower limits first quartile (25th percentile); whiskers = 1.5 times the interquartile range; points =  
785 outliers (>1.5 times the interquartile range).

786 Figure 3. Ecological status (green=good, yellow=moderate, orange= poor, red =bad) of the six  
787 analyzed lagoons according to the different biological indices (MaQI, M-AMBI, M-bAMBI, ISD)  
788 from 2008 to 2010 (See paragraph 2.4 for thresholds of each index).

789 Figure 4. Boxplot showing percentage of ecological groups calculated on abundances (A),  
790 ecological groups calculated on biomass (B), size classes used for ISD calculation (C), and W  
791 statistic (D).

792 Figure 5. Redundancy analysis showing the relations between environmental factors (blue arrows)  
793 and ecological groups (A) calculated on abundances (AB) and biomass (BIO), size classes (B), and  
794 biotic indices (C) (red labels). Total inertia: 35.91 (A), 78.283 (B), 1.117 (C); Eigenvalues displayed  
795 in figure axes.

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