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ScienceDirect
Estuarine, Coastal and Shelf Science 78 (2008) 1–13
ESTUARINE COASTAL AND SHELF SCIENCE
www.elsevier.com/locate/ecss

Impacts of urban wastewater discharge on seagrass meadows (*Zostera noltii*)

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Received 6 August 2007; accepted 14 November 2007
Available online 19 November 2007

Abstract

The abiotic disturbance of urban wastewater discharge and its effects in the population structure, plant morphology, leaf nutrient content, epiphyte load and macroalgae abundance of *Zostera noltii* meadows were investigated in Ria Formosa coastal lagoon, southern Portugal using both univariate and multivariate analysis. Four sites were assessed, on a seasonal basis, along a gradient from a major Waste Water Treatment Works (WWTW) discharge to a main navigation channel. The wastewater discharge caused an evident environmental disturbance through the nutrient enrichment of the water and sediment, particularly of ammonium. *Zostera noltii* of the sites closest to the nutrient source showed higher leaf N content, clearly reflecting the nitrogen load. The anthropogenic nutrient enrichment resulted in higher biomass, and higher leaf and internode length, except for the meadow closest to the wastewater discharge (270 m). The high ammonium concentration (158–663 µM) in the water at this site resulted in the decrease of biomass, and both the leaf and internode length, suggesting a toxic effect on *Z. noltii*. The higher abundance of macroalgae and epiphytes found in the meadow closest to the nutrient source may also affect the species negatively. Shoot density was higher at the nutrient-undisturbed site. Two of the three abiotic processes revealed by Principal Component Analysis were clearly related to the WWTW discharge, a contrast between water column salinity and nutrient concentration and a sediment contrast between both porewater nutrients and temperature and redox potential. A multiple regression analysis showed that these abiotic processes had a significant effect on the biomass-density dynamics of meadows and on the overall size of *Z. noltii* plants, respectively. Results show that the wastewater discharge is an important source of environmental disturbance and nutrients availability in Ria Formosa lagoon affecting the population structure, morphology and N content of *Z. noltii*. This impact is spatially restricted to areas up to 600 m distant from the WWTW discharge, probably due to the high water renewal of the lagoon.

Keywords: wastewater; urban effluents; nutrient enrichment; disturbance; seagrass; *Zostera noltii*; Ria Formosa

1. Introduction

Seagrass declines have been reported worldwide, whether natural or human-induced. Most of these declines are attributed to anthropogenic disturbances, such as eutrophication, toxic pollutants and mechanical damages (Short and Wyllie-Echeverria, 1996). Urban and industrial development, as well as intensive agriculture, in proximity of coastal areas have resulted in the increase of nutrient inputs to the near shore and estuarine ecosystems all over the world. Eutrophication of coastal waters may follow the increased nutrient loads, promoting the deterioration of water quality (Short and Wyllie-Echeverria, 1996). The reduction of water transparency limits the light penetration into the water column generating an important threat to seagrasses by reducing the light availability for photosynthesis. Although direct toxic effects of nutrients have been demonstrated (Burkholder et al., 1992; Van Katwijk et al., 1997; Brun et al., 2002), seagrass declines seem to be primarily related to the indirect effects associated with eutrophication, namely the responses of other primary producers to the nutrient loads

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doi:10.1016/j.ecss.2007.11.005

(Hemminga and Duarte, 2000). Macroalgae, phytoplankton and epiphytes proliferate as nutrient inputs increase (Borum, 1985; Tomasko and Lapointe, 1991; Frankovich and Fourqurean, 1997; Wear et al., 1999; Hauxwell et al., 2003), because they can rapidly assimilate the nutrients from the water column (Duarte, 1995). The overgrowth of macroalgae and epiphytes leads to the shading and suffocation of seagrass meadows, contributing to seagrass declines (Duarte, 1995; Hughes et al., 2004; Lapointe et al., 2004). Moreover, other indirect effects associated with eutrophication, such as sediment anoxia may also be detrimental for seagrasses (Terrados et al., 1999).

As the other primary producers, seagrasses also respond to the increase of nutrients. However, the lower rates of nutrients uptake under abundant nutrient supply makes seagrasses inferior competitors compared with macroalgae (Duarte, 1995). In a meta-analysis approaching the nutrient effects on seagrasses, Hughes et al. (2004) identified the biomass as one of the seagrass parameters that respond positively to the *in situ* nutrient enrichment of the sediment (particularly the aboveground biomass). The water column nutrient additions had strong negative effects on seagrass biomass, because it also results in increased epiphyte biomass (Hughes et al., 2004). On the other hand, the seagrass nutrient content reflects to a certain extent the nutrient availability in marine ecosystems. Higher nutrient concentrations have been related with higher nutrient contents in plant tissues (Borum et al., 1989; Duarte, 1990; Fourqurean et al., 1992; Udy and Dennison, 1997b; Lee et al., 2004). Even though the effects of increased nutrients on seagrasses have been extensively documented by experimental and field studies, the direct impact of an urban wastewater effluent on seagrass meadows was never assessed.

Zostera noltii is a small seagrass species distributed along the intertidal and subtidal areas of the Northern and Western Europe, Mediterranean Sea and North-West Africa (Den Hartog, 1970). The decline of *Z. noltii* meadows has been reported in the Wadden Sea (Philippart and Dijkema, 1995), the southern coasts of Spain (e.g. Palmones River estuary; Niell et al., 1996; Hernández et al., 1997) and in the Portuguese coast (e.g. Mondego estuary; Oliveira and Cabeçadas, 1996; Cardoso et al., 2004), mostly as a consequence of eutrophication. In Ria Formosa lagoon, southern Portugal, *Z. noltii* is the most abundant seagrass, covering large areas of the lower intertidal. This species plays an important role in the lagoon productivity (Santos et al., 2004). Even though this system constitutes a natural park, the conservation status of this species in the lagoon is unknown, due to the lack of long-term monitoring studies. Thus, the assessment of the effects caused by the increasing anthropogenic nutrient load assumes a major importance in order to prevent potential seagrass declines.

The main objective of this study was to determine the relationships among the urban wastewater discharge and the population structure, plant morphology, nutrient content, epiphyte load and macroalgae abundance of *Zostera noltii* meadows of Ria Formosa lagoon, along a gradient from a major urban wastewater discharge to the main navigation channel of the lagoon.

2. Materials and methods

2.1. Study sites

Ria Formosa lagoon is a mesotidal system located in the southern coast of Portugal (Fig. 1). The lagoon has a high spring tide surface area of 84 Km^2, with an exposed intertidal area of approximately 80%. The lagoon is separated from the Atlantic Ocean by a system of five sand barrier islands and six inlets. In each tidal cycle about 50–75% of the water in the lagoon is renovated. The tidal amplitude ranges from 3.50 m on spring tides to 1.30 m on neap tides, and salinity ranges from 35.5 to 36 along the year. Four continuous *Zostera noltii* meadows of similar bathymetry, located in the lower intertidal, were selected along a tidal creek from a Waste Water Treatment Works (WWTW) effluent to a main navigation channel of Ria Formosa (Fig. 1). The period of meadow emersion during low tide is small (less than one hour), and after that period, the flooding water rapidly covers the meadows. Site 1 was the *Z. noltii* meadow closest to the WWTW discharge (270 m), while site 4 was located in the main channel where there

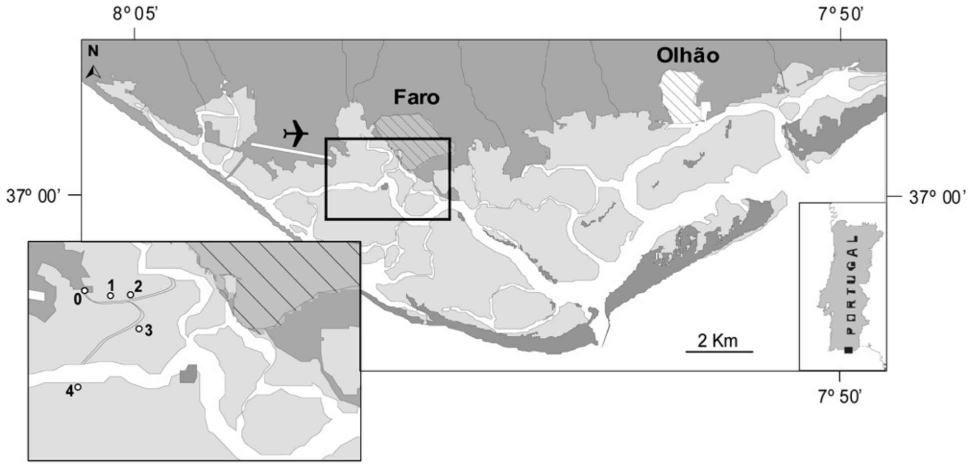

Fig. 1. Map of Ria Formosa, southern Portugal, with the location of the sampling sites from the wastewater effluent (site 0) to the main navigation channel (site 4).

was no influence of the wastewater effluent (1500 m). Sites 2 and 3 were 520 m and 610 m distant from the WWTW discharge, respectively. Water circulation flows from site 1 to site 4 during ebb tide and from site 4 to site 1 during flood tide. The WWTW has a 12,000 e.h. and is characterised by a sewage flow of ca. 0.12 $m^3 s^{-1}$ of domestic sewage (CCDR Algarve, http://www.ccdr-alg.pt/ccr/index.php). No meaningful input of freshwater or industrial waste occurs in this area.

2.2. Environmental variables

Environmental parameters of water and sediment were determined seasonally, in July 2001 (summer), November 2001 (autumn), February 2002 (winter) and May 2002 (spring), at the *Zostera noltii* sampling sites (sites 1−4, Fig. 1). The effluent water of the WWTW was also characterised (site 0, Fig. 1). Water salinity, temperature and pH were recorded *in situ* in each site. Three water samples (100 ml each) were collected in each site, at low tide, filtered (Whatman cellulose acetate filters, 0.45 μm pore size) and frozen until nutrient analysis. Ammonium was determined using a spectrophotometric method (Solorzano, 1969), while nitrates + nitrites and phosphates were determined with an Autoanalyser system (Skalar, Sans Plus).

The sediment measurements were done in the first 5 cm of the sediment surface, within the *Zostera noltii* rhizosphere. The redox potential (Eh) was measured *in situ* at 5 cm depth with a metal electrode (Russell RL100). Sediment cores were collected during low tide, and sliced *in situ*. A total of six minicores (3.5 cm of diameter) were pooled for each sample (three samples per site), in order to reduce the effect of the nutrient patchiness in the sediment. The sediment samples were centrifuged (3000 rpm, 20 min at *in situ* sediment temperature) and the supernatant water was filtered (Whatman acetate cellulose filters, 0.45 μm pore size) and frozen until nutrient analysis. Sediment samples were then homogenised and a subsample was dried (48 h at 60 °C) and combusted (4 h at 450 °C) to determine the organic matter content. In summer and winter, another sediment subsample was dried and analysed to determine the C, N and P content of the sediment. The C and N content was analysed using a Carlo-Erba elemental analyser (EA1108). To measure the P content, the dried sediment was burned for one hour at 550 °C, 0.2 M HCl was added and then incubated for one hour at 100 °C. The extracted orthophosphate was analysed using spectrophotometric analysis (Koroleff, 1983). The remaining sediment was analysed only once, in summer, to determine the grain size (% of sand and % of silt plus clay) following Erftemeijer and Koch (2001).

2.3. Seagrass characteristics

Five randomly distributed samples of *Zostera noltii* were collected seasonally from each site with a 12 cm diameter core. The number of shoots was counted in each sample to estimate shoot density. The leaf length, the leaf width, the leaf number per shoot and the sheath length was measured from intact shoots, the internode length and diameter was measured from intact rhizome internodes and the root length was recorded from intact roots (accuracy of 1.0 mm for leaf, sheath, internode and root length and of 0.01 mm for leaf width and rhizome diameter). The above and belowground biomass of *Z. noltii* was determined by drying the samples at 60 °C for 48 h. *Zostera noltii* leaves were analysed to determine the C, N and P content, both in summer and in winter, using the same methods as described above for the sediment. The elemental ratios of C:N and C:P were calculated on a mol:mol basis.

2.4. Macroalgae and epiphytes

The macroalgae present in each *Zostera noltii* core was separated and dried at 60 °C during 48 h. In summer, 15 shoots were randomly taken from each sample to assess the abundance of epiphytes on *Z. noltii* leaves. All epiphytes were smoothly scrapped from the leaves and collected on Whatman GF/F filters. Epiphytes and shoots were separately dried at 60 °C for 48 h, to estimate the percentage of epiphytes on a dry weight basis.

2.5. Statistical analysis

2.5.1. Univariate analysis

Significant differences in data sets were investigated using one- or two-way ANOVA, after testing for homogeneity of variances and normality of distributions. When necessary, variables were log transformed to fit ANOVA assumptions. When ANOVA indicate significant differences among sites and/or seasons, Tukey's multiple comparison test was applied to determine which site(s) and/or season(s) were significantly different from each other. When ANOVA assumptions were not verified, comparison of data sets was performed using the non-parametric test of Kruskal−Wallis. Significance levels were tested at $p < 0.05$ (Sokal and Rohlf, 1995).

2.5.2. Multivariate analysis

Principal Component Analysis (PCA) was used to analyse both abiotic (12 variables) and biotic (11 variables) data sets. The significance of the statistics performed to decide which eigenvectors to use as principal components (PCs) and which variables were correlated to each PC were estimated using the permutation tests of Monte Carlo methods (Manly, 2006). To select which PCs to analyze, both a test for the equality of roots (Jackson, 1991) and a test for the significance of the eigenvalues were performed in the original data set and in 10,000 randomly permutated data sets. The null hypothesis of each PC describing only random variation was tested by comparing the magnitude of the statistic of the original data set with those obtained from the permutated data sets. Both the Scree plot and the Broken Stick techniques were used to confirm the results obtained. To assess the significance of the loadings of each variable in each selected PC two statistics were used, the correlation of each variable with each PC and a modified version of this statistic. As the first statistic overestimates the loadings of non-significant PCs relative to their eigenvalues:

$$\frac{u_{ij}\sqrt{\lambda_i}}{s_j}$$

where u_{ij} is the loading of the variable j in principal component i, λ_i is the eigenvalue of the principal component i and S_j is the standard deviation of variable j; the loadings and eigenvalues were squared in order to make the statistic less sensitive to high loadings generated by random error:

$$\frac{u_{ij}^2\lambda_i^2}{s_j}$$

In this way, only high loadings of PCs with high eigenvalues are likely to be considered significant. The *z*-scores of each sample in each selected PC were calculated to analyse spatial and temporal patterns of the processes identified by each PC. The *z*-score is the value of each sample in the new metric of each PC and is a measure of the sample dispersion (given by its residual in each variable) weighted by the contributions of the respective variables to the PC (given by their loadings). Multiple regressions of each selected biotic PC (dependent variable) on the selected abiotic PCs (independent variables) were performed to determine which environmental processes are significantly related to the biotic processes determined by the PCA analysis.

3. Results

3.1. Univariate analysis

3.1.1. Environmental variables

The concentration of nutrients in the water (Fig. 2A) showed an evident and significant gradient from the wastewater effluent (site 0) to the main channel (site 4, Table 1). The wastewater effluent (site 0) provided an ammonium input that ranged between 571 ± 56 μM (summer) and 1801 ± 16 μM (spring). At the seagrass meadows along the effluent gradient, ammonium was the most available nutrient in the water column, varying from 158–663 μM at site 1 to 0.6–4.2 μM at site 4. The nutrients in the sediment porewater also followed the gradient originated by the wastewater discharge (Fig. 2B, Table 1), except for site 1 that showed intermediate values. There were higher nutrient concentrations at site 2 (80–217 μM for ammonium, 0.3–7.0 μM for nitrates + nitrites, 22–52 μM for phosphates) and lower concentrations at site 4 (12–38 μM for ammonium, 0.2–0.9 μM for nitrates + nitrites, 2.4–13.9 μM for phosphates).

The organic matter content of the sediment (Table 2) at sites 2 and 3 was significantly higher than at sites 1 and 4. The redox potential showed more negative values at site 2, although not significantly different from the others sites (Table 2). Site 4 showed a significantly different grain size of the sediment (Table 2), with more sand (33%) and less fine sediments (66%), than the other sites. The C, N and P content of the sediment was not significantly different between summer and winter. The C content of the sediment of site 2 (3% DW) was significantly higher than in the other sites, while the N content of site 4 sediment (0.16% DW) was significantly lower (Table 2). No significant differences were detected in the sediment P content among sites.

3.1.2. Seagrass characteristics

The C, N and P content of *Zostera noltii* leaves (Fig. 3) was significantly higher in winter than in summer. The N content of *Z. noltii* leaves from site 4 was significantly lower than from sites 1 to 3. However, no significant differences were found in C and P leaf content among the study sites (Table 3). The C:N ratio of *Z. noltii* leaves from site 4 was significantly higher than from sites 1 and 3 (Fig. 3D), while the C:P ratio did not vary significantly among the study sites (Fig. 3E). Both the C:N and C:P ratios were significantly higher in summer than in winter.

The total biomass of *Zostera noltii*, as well as the above and belowground biomass, showed significant effects of site and season (Table 3). The total biomass at site 1 was significantly lower than at the other sites (Fig. 4A). This site showed the lowest total biomass along the sampling period (always less than 210 g DW m^{-2}). The seasonal variation of biomass was much higher in site 2 (127–429 g DW m^{-2}) than in other sites (119–210 g DW m^{-2} for site 1, 172–372 g DW m^{-2} for site 3, and 229–310 g DW m^{-2} for site 4). The seasonal variation of the aboveground biomass followed the trend of total biomass, increasing in summer and approaching belowground biomass in winter. Belowground biomass was fairly constant throughout the sampling period. In general, belowground biomass was lower than aboveground biomass.

Shoot density was significantly higher at site 4 relative to sites 1 and 3 (Fig. 4B). In general, site 4 showed significantly higher density throughout the sampling period, particularly in summer, when density reached more than 13,000 shoots m^{-2}. A significant effect of site and season on the leaf and internode length was found (Table 3). Both measures were significantly higher at site 2 and lower at site 4 (Figs. 4C,D). All sites showed the longest leaves in autumn (194 mm for site 1, 328 mm for site 2, 285 mm for site 3 and 235 mm for site 4), whereas longer internodes were observed in spring (12.9 mm for site 1, 17.3 for site 2, 17.6 mm for site 3 and 9.2 mm for site 4).

3.1.3. Macroalgae and epiphytes

The biomass of macroalgae within *Zostera noltii* meadows, mainly Ulvales, was significantly higher at site 1 (Fig. 5A), particularly in spring, when these opportunistic algae peaked. Epiphytes on *Z. noltii* leaves, mostly diatoms, showed significantly higher percentage at site 1 (6.8%) relative to sites 2 and 4 (0.8 and 0.5%, respectively; Fig. 5B).

3.2. Multivariate analysis

Three meaningful PCs were selected both for the abiotic and biotic data sets (Tables 4 and 5). The three abiotic PCs

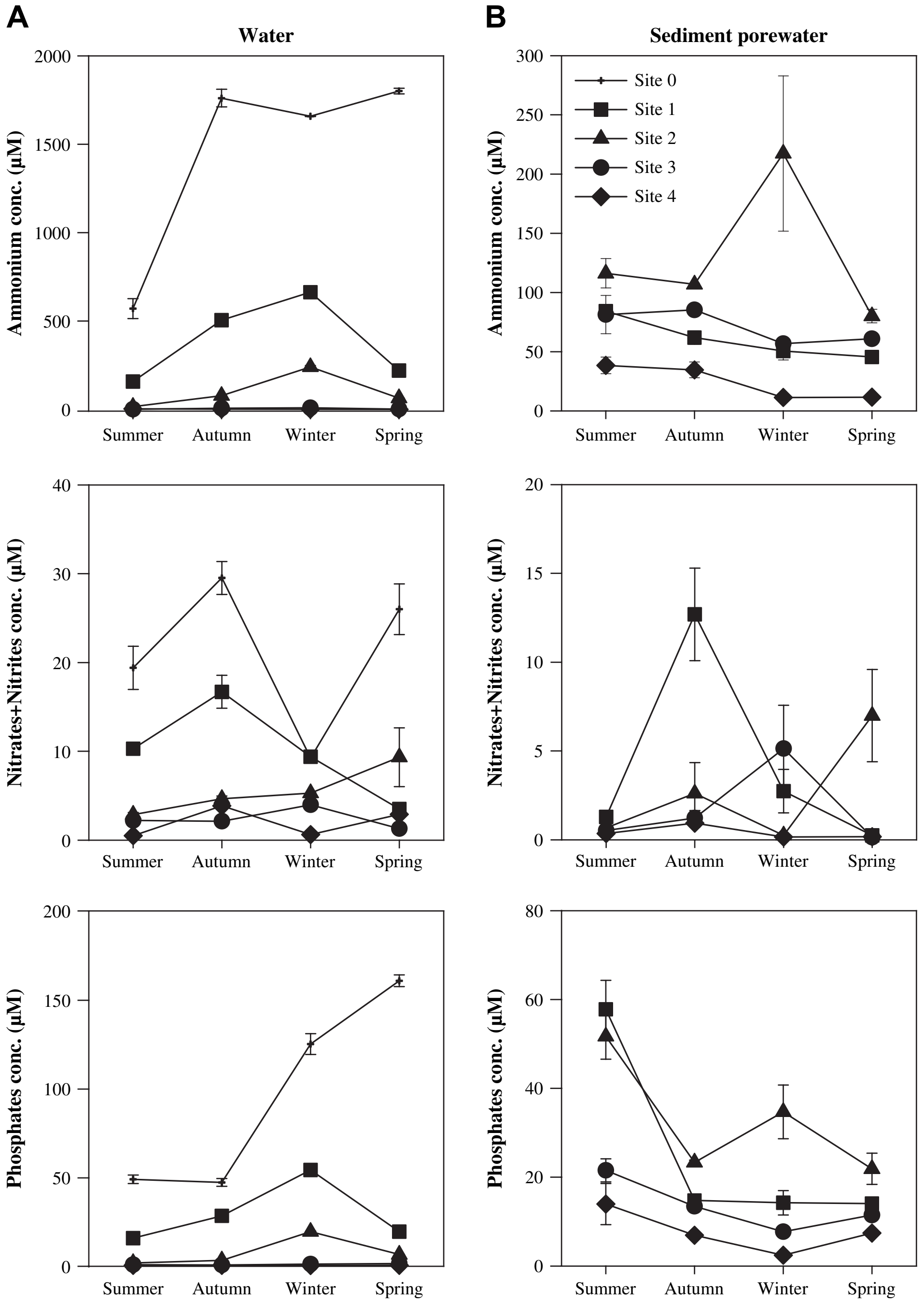

Fig. 2. Seasonal and spatial variation of nutrients (ammonium, nitrates + nitrites and phosphates) in the (A) water (site 0 to 4) and in the (B) sediment porewater (site 1–4). Error bars represent standard errors.

account for 76.8% of total variation (Table 4). The first one revealed a process where nutrients (ammonium, nitrates + nitrites and phosphates in the water column and nitrates + nitrites in the sediment porewater) are negatively related to the water column salinity (Table 4). This PC exhibits a clear seasonality (Fig. 6A), indicating high inputs of nutrient enriched freshwater in autumn and winter, when salinity is lower (lower *z*-scores), and low inputs in spring and summer (higher *z*-scores). The seasonal pattern is very evident at site 1 and fades away to site 4, where no meaningful seasonal variation was found. This indicates that the freshwater input with high nutrients originates from the wastewater discharge. The increase of the *z*-scores from site 1 to site 4 also supports this hypothesis as it reflects the increased influence of the lagoon water with higher salinity and lower nutrient concentrations. The second PC reveals a sediment biogeochemical process where both the sediment porewater nutrients (ammonium and phosphate) and temperature (water column) are negatively related to the sediment redox potential (Table 4). When the temperature and the porewater ammonium and phosphate are higher, the redox potential is lower (a strong reducing environment). This process also exhibited a clear seasonal pattern

Table 1
Statistical results and significance of ANOVA (F) examining the effects of site and season on the nutrient concentrations of the water and sediment porewater. (**) $p < 0.01$, (***) $p < 0.001$

	Site	Season	Site × Season
Water			
Ammonium	5027.3***	448.2***	232.7***
Nitrates + Nitrites	144.9***	17.3***	13.3***
Phosphates[a]	6424.5***	319.6***	78.6***
Sediment porewater			
Ammonium[a]	113.1***	15.8***	6.9***
Nitrates + Nitrites	7.4***	6.3**	8.4***
Phosphates	51.4***	44.1***	7.7***

[a] Log transformed data prior ANOVA.

(Fig. 6B) characterized by a summer high of ammonium, phosphate, reducing power and temperature (higher z-scores) and the opposite in autumn, winter and spring (lower z-scores). Both the z-scores and the seasonal pattern are high up to site 3, decreasing to site 4, where no meaningful seasonal variation was found. This suggests that the influence of the urban effluent is evident up to site 3 and not at site 4. The third PC reveals a contrast between the organic matter in the sediment and temperature (sediment and water column, Table 4). In summer and spring, when temperatures are higher, the sediment organic matter was lower (higher z-scores), probably due to higher remineralization (Fig. 6C). Contrary to the other PCs, there is no evident decrease of the z-scores and the seasonal pattern from site 1 to site 4, indicating that this process is not related to the urban effluent discharge.

Three PCs were also selected for analysis for the biotic data set (Table 5). The three PCs together accounted for 79.7% of total variation. The first PC represents the diameter of *Zostera noltii* rhizomes and the length of roots, which are negatively related to the length of leaves (Table 5). The z-score analysis showed that the relative magnitude of the belowground components was higher in spring whereas the leaf length was more important in autumn. This seasonal pattern was consistent in all sites, even though it was less pronounced in site 1 where the relative magnitude of the rhizome diameter and root length was higher (Fig. 7A). The second PC represents the overall size of *Z. noltii* plants, where a positive relationship among the leaf metrics (leaf width, sheath length and leaf length, this one with $p = 0.07$) and the rhizome internode length was revealed (Table 5). The overall size of the plants did not vary much spatially or seasonally (Fig. 7B). The third PC reveals a positive relationship between *Z. noltii* total biomass (above and belowground) and density (Table 5). The analysis of the z-scores shows that, in general, the total biomass and density increased along the sites 1–4 and that these variables have a high in summer and a low in winter (Fig. 7C). Exceptions to this pattern are the autumn low in site 1 and the spring high in site 2.

The three main biological patterns described for *Zostera noltii* populations by the three significant biotic PCs were significantly related to the three meaningful abiotic processes as revealed by the multiple regression analysis (Table 6). The relative sizes of rhizomes/roots versus leaves, described by the first PC is highly correlated ($p = 0.001$) to the third abiotic PC, which represents the temperature contrast with sediment organic matter. When temperatures are higher and the organic matter content of the sediment is lower, the rhizome diameter and the root length of *Z. noltii* are higher and their leaves are shorter. The process described by the second biotic PC, which represents the overall size of the plants, is correlated (with $p = 0.08$) to the second abiotic PC, which represents the contrast between both the sediment porewater nutrients (ammonium and phosphate) and temperature (water column) with the sediment redox potential. This suggests that the size of *Z. noltii* plants is more influenced by within sediment ammonium and phosphate, rather than other variables such as water column nutrients. Finally, the biological process described by the third biotic PC, which represents the biomass-density dynamics of *Z. noltii* populations, was significantly correlated ($p = 0.04$) to the process described by the first abiotic PC, which is clearly related to the urban wastewater discharge, as it describes a pattern of high nutrients and low salinity in the water.

4. Discussion

The urban wastewater discharge into the Ria Formosa lagoon establishes an evident environmental disturbance, which was related to changes in the *Zostera noltii* population

Table 2
Sediment characteristics of *Zostera noltii* sampling sites. Values represent annual means ± standard errors of organic matter (OM, $n = 12$), redox potential (Eh, $n = 4$) and elemental contents (CNP, $n = 6$), except for grain size ($n = 3$). Different letters denote significant differences among sites in one-way ANOVA (F values). (*) $p < 0.05$, (***) $p < 0.001$, (^{ns}) not significant

	Site 1	Site 2	Site 3	Site 4	F
OM (% DW)	6.3 ± 0.3^a	8.6 ± 0.3^b	8.1 ± 0.3^b	5.2 ± 0.2^a	24.67***
Eh (mV)	−227 ± 18	−309 ± 21	−234 ± 38	−192 ± 30	3.14^{ns}
Grain size					
Sand (% DW)	13.5 ± 2.3^a	18.2 ± 0.3^a	12.3 ± 2.1^a	33.4 ± 3.1^b	19.11***
Silt + Clay (% DW)	86.5 ± 2.3^a	81.6 ± 0.5^a	87.6 ± 2.2^a	65.8 ± 3.0^b	21.23***
Nutrient content					
C (% DW)	2.29 ± 0.14^a	3.01 ± 0.18^b	2.22 ± 0.19^a	2.14 ± 0.08^a	6.88*
N (% DW)	0.24 ± 0.01^a	0.26 ± 0.00^a	0.25 ± 0.01^a	0.16 ± 0.01^b	25.16***
P (% DW)	0.23 ± 0.01	0.22 ± 0.01	0.26 ± 0.01	0.25 ± 0.01	2.95^{ns}

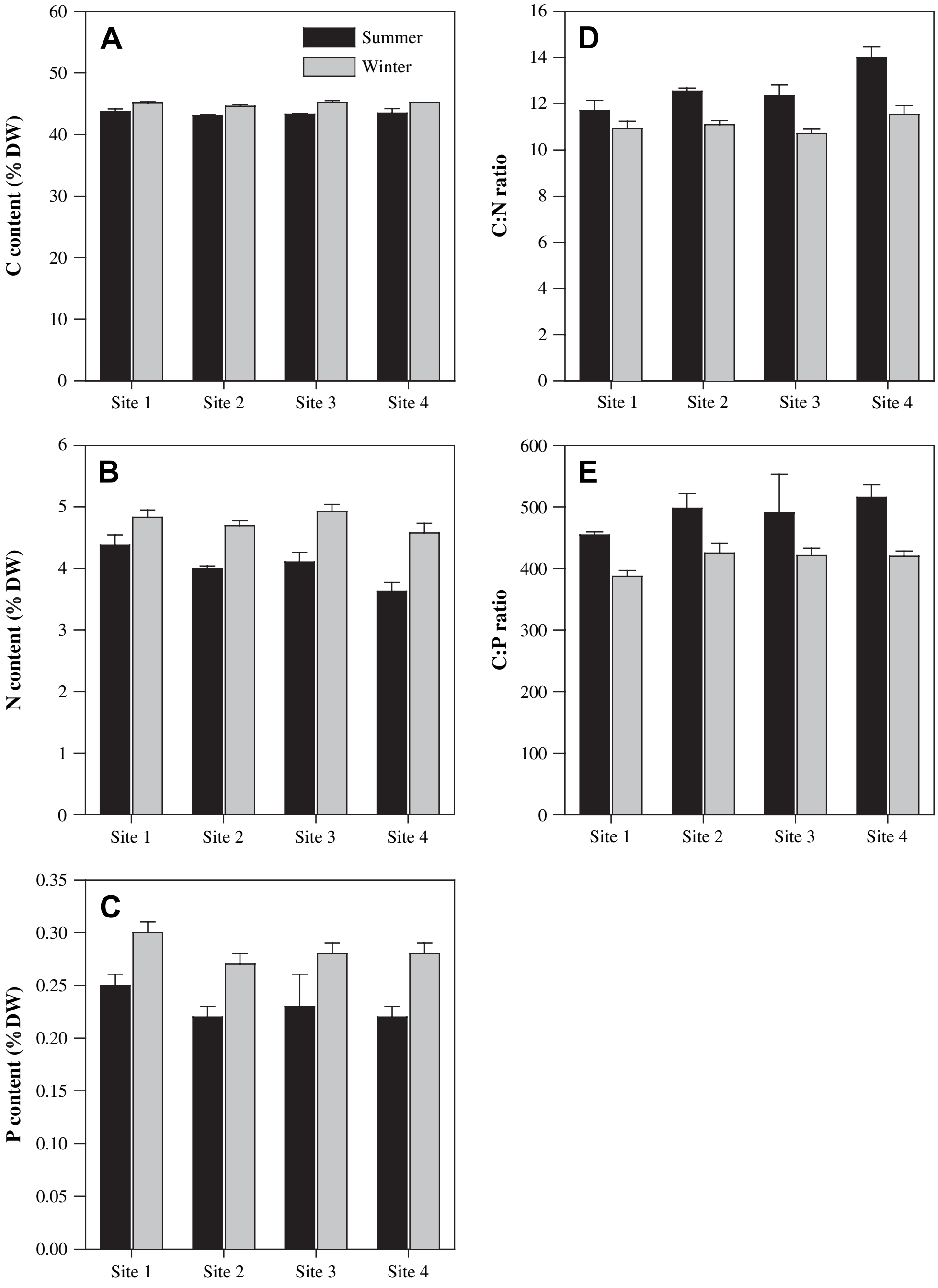

Fig. 3. Seasonal and spatial variation of (A) carbon content, (B) nitrogen content, (C) phosphorous content, (D) carbon:nitrogen ratio and (E) carbon:phosphorus ratio of *Zostera noltii* leaves. Error bars represent standard errors.

structure, plant morphology and tissue nutrient contents. The coupling between the environmental disturbance and the biological processes operating within *Z. noltii* populations was clearly showed by the significant correlations found between the main abiotic and biotic processes revealed by the multivariate analysis. The high availability of nutrients throughout the year, both in the water and in the sediment porewater, reflected the effects of the wastewater effluent. The abiotic process explaining more variation (35%) was clearly related to the wastewater discharge (Table 4), as it described the negative relationship between nutrients and salinity in the water and a spatial pattern that faded away with the distance to the wastewater effluent. This supports the fact that the wastewater discharge is the major source of water column nutrients. The availability of nutrients decreased with the distance to the WWTW in a way that the *Z. noltii* meadow near the discharge was exposed to ammonium concentrations in the water of up to three orders of magnitude higher than the distant meadow. As well, the sediment of the sites along the wastewater creek was much richer in nutrients, in organic matter and in silt plus clay than the undisturbed site (Table 2). This reflects both the effluent inputs and the lower hydrodynamic forces of the inner

Table 3
Statistical results and significance of ANOVA (F) or Kruskal–Wallis (H) tests examining the effects of site and season on plant variables. (**) $p < 0.01$, (***) $p < 0.001$, (^{ns}) not significant

	Site	Season	Site × Season
Total biomass[a]	$F = 29.04$***	$F = 19.78$***	$F = 5.81$***
Density	$F = 23.11$***	$F = 40.43$***	$F = 11.16$***
Leaf length	$H = 178.14$***	$H = 291.00$***	–
Internode length	$H = 109.37$***	$H = 179.90$***	–
Leaf C content	$F = 1.48^{ns}$	$F = 52.83$***	$F = 0.24^{ns}$
Leaf N content	$F = 6.06$**	$F = 67.54$***	$F = 1.47^{ns}$
Leaf P content	$F = 3.30^{ns}$	$F = 46.71$***	$F = 0.24^{ns}$
Leaf C:N ratio	$F = 7.34$**	$F = 44.02$***	$F = 2.08^{ns}$
Leaf C:P ratio	$F = 2.34^{ns}$	$F = 28.38$***	$F = 0.22^{ns}$

[a] Log transformed data prior ANOVA.

sites compared to the site located on the main navigation channel. During the period of this study, an unexpected artificial input of sediments originated from works related to the airport expansion towards the lagoon may have altered the sediment condition of site 1. This probably masked the influence of the wastewater discharge on the sediments of this site, which showed lower nutrient concentrations and organic matter contents than contiguous sites (Fig. 2B, Table 2). Nevertheless, the sediment biogeochemical process described by the second abiotic PC, which describes the positive relationship between the nutrients and anoxia condition of the sediment, was clearly related to the wastewater discharge. This effect was evident up to site 3, about 600 m distant from the WWTW, but not in site 4 (Fig. 6B), about 1500 m distant from the WWTW, revealing the extent of the WWTW disturbance into the Ria Formosa lagoon.

As a consequence of the environmental nutrient enrichment caused by the wastewater discharge, both the *Zostera noltii* leaves and the sediment organic matter of the nearby sites showed significantly higher N content, revealing a physiological response of the biota to the N input. The tissue nutrient content of seagrasses is a good indicator of the environmental nutrient enrichment (Udy and Dennison, 1997b), since it reflects the local nutrient availability, as observed in this study. In particular, *Z. noltii* may be a good biological indicator of nutrient loading to coastal ecosystems, as this is a fast growing species (Marbà and Duarte, 1998) and thus its internal nutrient contents rapidly reflect the environmental conditions. The seasonal variation of the nitrogen and phosphorous inputs by the wastewater discharge was also reflected in the N and P content of *Z. noltii* leaves (Fig. 3), which were higher in winter when there were higher inputs of nutrients (Fig. 2A). However, the higher tissue nutrient content during winter may also reflect

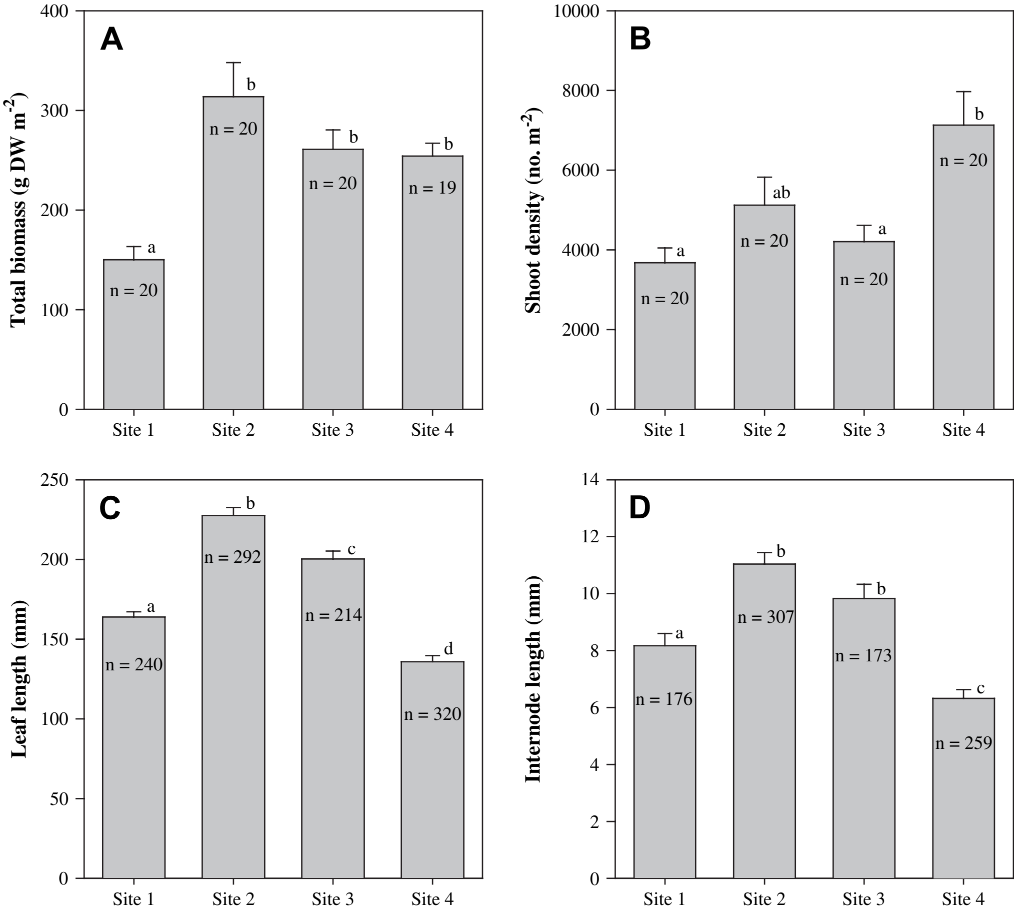

Fig. 4. Spatial variation of (A) total biomass, (B) shoot density, (C) leaf length and (D) internode length of *Zostera noltii* (annual means). Error bars represent standard errors. Different letters denote significant differences.

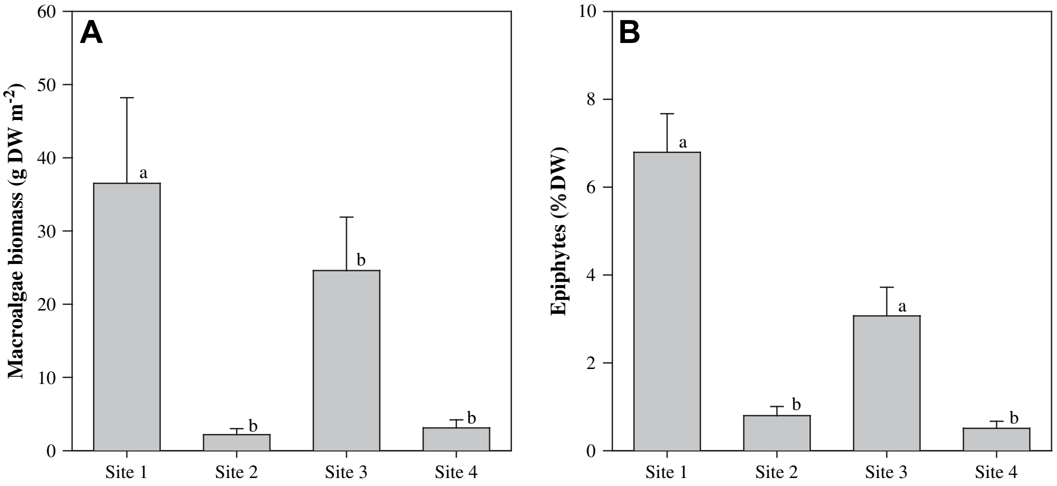

Fig. 5. Algae abundance in *Zostera noltii* meadows. (A) Macroalgae biomass (annual mean) and (B) epiphyte load on seagrass leaves in summer. Error bars represent standard errors. Different letters denote significant differences.

the species growth seasonality, as the tissue contents is usually highest during the low growing season (winter) and lowest during the high growing season (summer).

The high nutrient concentrations observed at site 1 (300 m distant from the WWTW discharge), both in the case of ammonium (158.3–663.4 μM) and of phosphate (15.8–54.2 μM), may have toxic effects for *Zostera noltii* as suggested by the lowest biomass of the species throughout the sampling period. Brun et al. (2002) findings corroborate our observations as ammonium concentrations of 200 μM were shown to have inhibitory toxic effects on *Z. noltii* growth and survival. Ammonium toxicity was also demonstrated for *Zostera marina* at concentrations of 125 μM, with plants becoming necrotic within 2 weeks (Van Katwijk et al., 1997). The evidence of toxicity at site 1 was also reflected in the lower length of leaves and internodes. Similarly, *Z. marina* plants exposed to high ammonium concentrations (75 μM and 125 μM) were also generally smaller, with shorter leaves (Van Katwijk et al., 1997). The lowest *Z. noltii* biomass observed in winter at site 2, when nutrient concentrations were higher (240 μM of ammonium and 19 μM of phosphate), also suggests the effects of toxicity. Other possible sources of toxicity have been identified for seagrasses, such as anoxia (Terrados et al., 1999), sulphides (Borum et al., 2005) and pesticides (Bester, 2000). However, these may not be important in this study area. No significant differences were found for the sediment redox potential, a proxy for anoxia, among the study sites and the Ria Formosa lagoon has low sulphide levels in the sediment due to the natural iron abundance, which sinks sulphides in the sediment in the form of pyrite. On the other hand, no data is available on the pesticides levels of the WWTW discharge, but they should not have high concentrations of pesticides, as these are mostly urban effluents.

The *Zostera noltii* population structure clearly reflected the effects of the urban wastewater discharge, as revealed by the significant correlation between the processes describing the biomass-density dynamics of *Z. noltii* populations (third biotic PC) and the wastewater discharge (first abiotic PC). The process describing the variation of biomass and density revealed a spatial pattern with *Z. noltii* biomass and density increasing with the distance to the wastewater effluent, but also a seasonal pattern, typical of temperate seagrasses, which seems to be amplified by the effect of the wastewater discharge (Fig. 7C).

On the whole, the shoot density of *Zostera noltii* meadows was higher at the nutrient-undisturbed site 4 (Fig. 4). The nutrient enrichment of the water column has been related to shoot density declines (Tomasko and Lapointe, 1991; Perez et al., 1994; Hauxwell et al., 2003), as the nutrient enrichment may promote the shoot mortality and/or reduce the shoot recruitment (Hauxwell et al., 2003). Van Katwijk et al. (1997) also found that high ammonium concentrations in water (125 μM) decreased the number of shoots for *Zostera marina* plants, as a consequence of increased necrosis and plant death. On contrary, the opposite response was reported for the sediment nutrient enrichment (Short, 1987; Van Lent et al., 1995; Lee and Dunton, 2000; Ibarra-Obando et al., 2004).

Table 4
Eigenvalues, % of explained variance (EV) and variables loadings to the eigenvectors of principal components (PC) extracted from the abiotic data correlation matrix. Significant PCs and their significant loadings are in bold

	Eigenvalue	EV (%)	NH4 sed.	NO3 sed.	PO4 sed.	OM sed.	Eh sed.	pH sed.	Temp. sed.	NH4 water	NO3 water	PO4 water	Sal. water	Temp. water
PC 1	4.21	35.1	−0.09	**−0.34**	−0.01	−0.11	−0.02	0.09	0.25	**−0.45**	**−0.42**	**−0.42**	**0.45**	0.21
PC 2	2.99	24.9	**0.39**	−0.05	**0.50**	0.23	**−0.50**	−0.30	0.27	0.04	0.12	0.07	0.04	**0.35**
PC 3	2.01	16.8	−0.38	0.06	0.10	**−0.54**	0.19	−0.02	**0.46**	0.16	0.18	0.17	−0.14	**0.45**

Table 5
Eigenvalues, % of explained variance (EV) and variables loadings to the eigenvectors of the principal components (PC) extracted from the biotic data correlation matrix. Significant PCs and their significant loadings are in bold

	Eigenvalue	EV (%)	Above biom.	Below biom.	Algae biom.	Shoot density	Leaf length	Leaf width	Leaf no.	Sheath length	Intern length	Intern diam.	Root length
PC 1	3.46	31.4	0.00	−0.01	0.29	0.18	**−0.40**	0.08	0.35	−0.36	0.33	**0.40**	**0.44**
PC 2	3.22	29.3	−0.30	0.15	−0.29	0.18	−0.32	**−0.51**	−0.07	**−0.38**	**−0.35**	−0.31	0.21
PC 3	2.10	19.1	**0.55**	**0.51**	−0.23	**0.52**	−0.09	0.20	−0.24	−0.04	0.04	−0.03	0.04

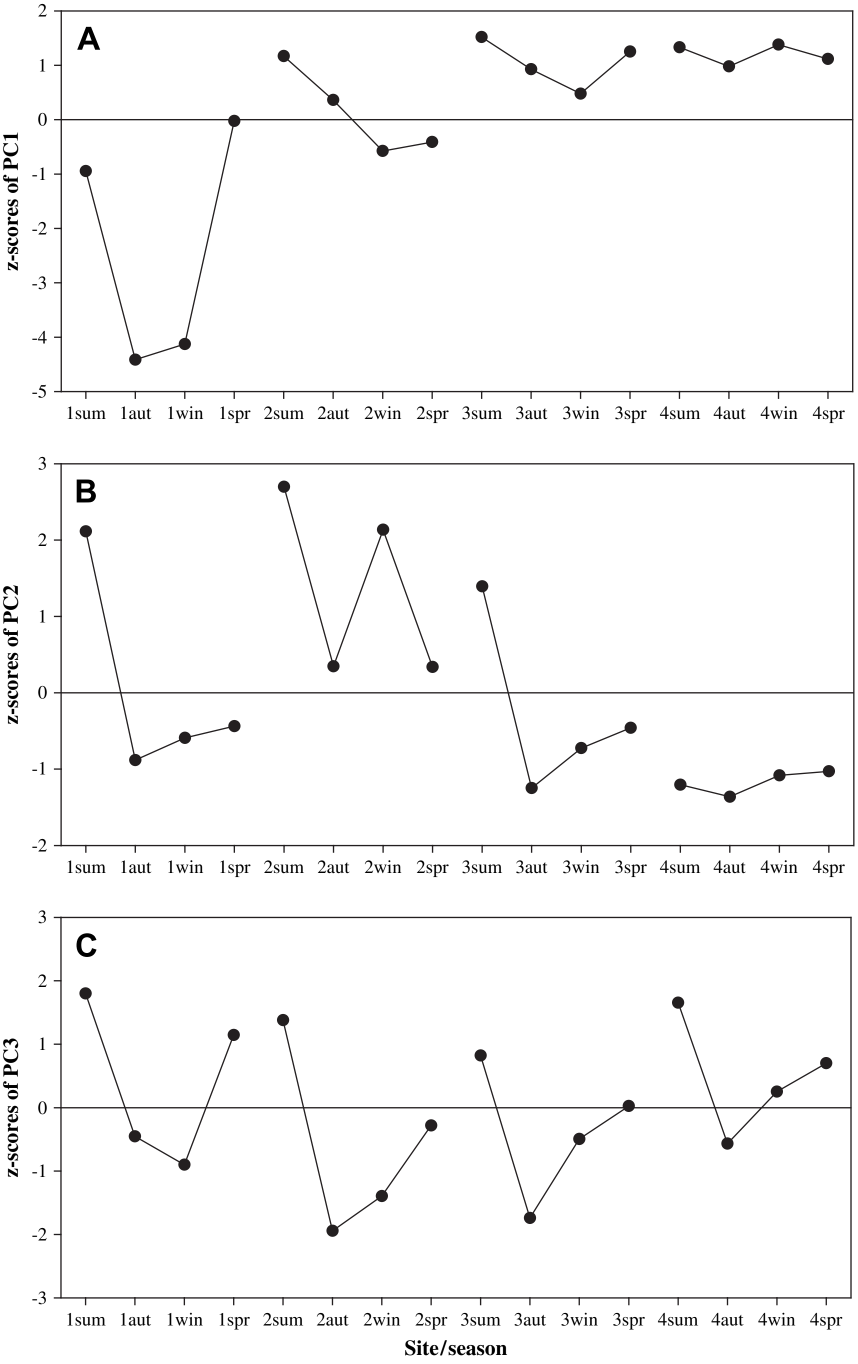

Fig. 6. Spatial and temporal variation of the *z*-scores of the (A) first PC, (B) second PC and (C) third PC for the abiotic data.

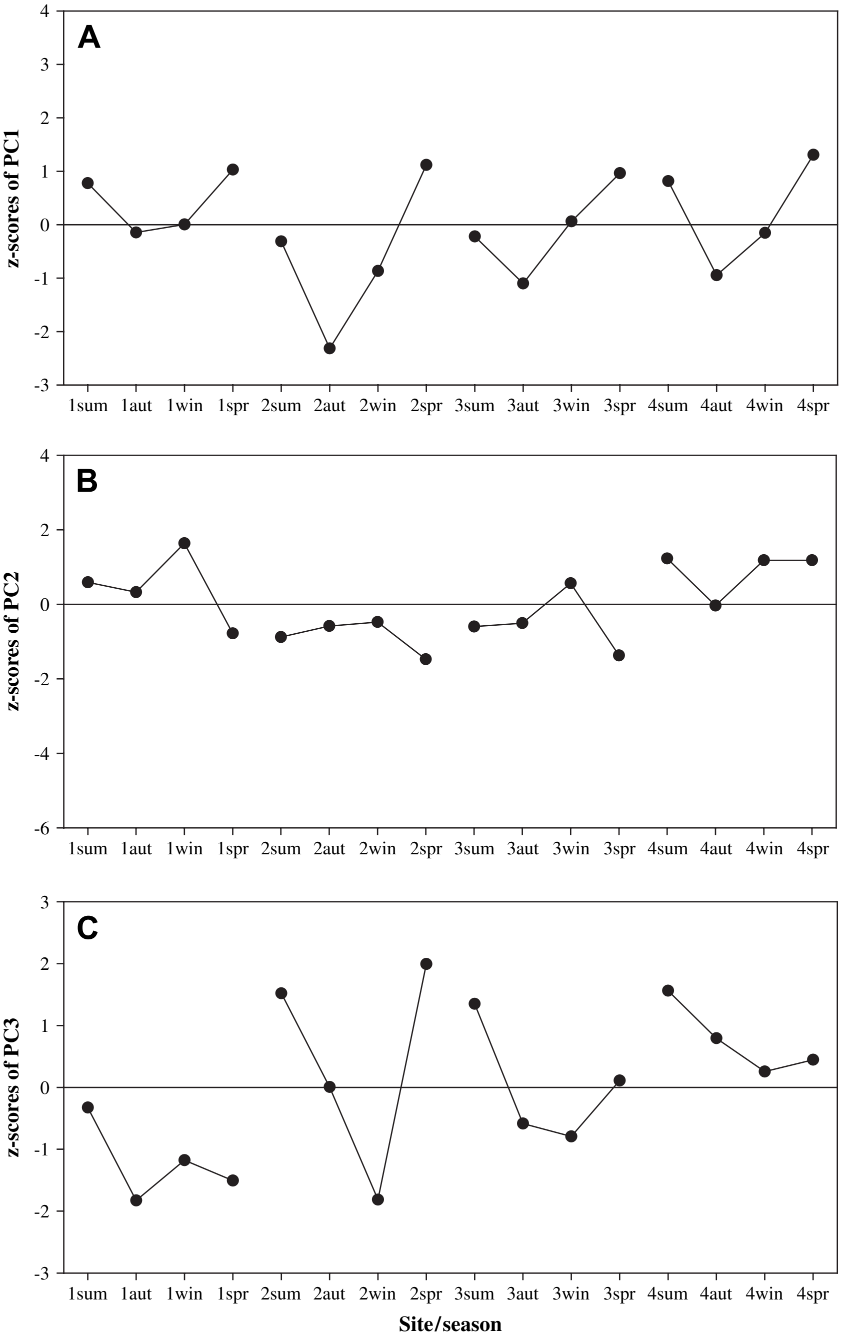

Fig. 7. Spatial and temporal variation of the *z*-scores of the (A) first PC, (B) second PC and (C) third PC for the biotic data.

In general, plant morphometry, such as leaf length and internode length were higher at the nutrient-enriched sites (Fig. 4), except at site 1, where the toxic effects of nutrients resulted in shorter leaves and internodes. While some studies have demonstrated that seagrass morphometry, such as canopy height or leaf length, increases with increasing nutrients availability (Perez et al., 1994; Udy and Dennison, 1997a,b; Udy et al., 1999; Lee and Dunton, 2000), others found no clear pattern of shoot height along nitrogen availability gradients (Lee et al., 2004). The morphological responses to nutrient enrichment may not show a clear trend, since seagrass growth is also influenced by other environmental variables, such as light, temperature, salinity, sediment sulphite or the concentration threshold for nutrient toxicity, which can distort the nutrients effect. This interaction was also observed in this study, as the morphometry of the plants, which was described by the two main processes occurring within *Zostera noltii* populations, was significantly related to different abiotic processes. The process describing the relative sizes of rhizomes/roots versus leaves (first PC) was correlated to the abiotic process describing the contrast between the temperature and the sediment organic matter (third abiotic PC), which is related to seasonal effects. On the other hand, the process describing the overall size of the plants (second PC) was related to the

Table 6
Results of multiple regression analysis between the *z*-scores of the biotic PCs (dependent variable) and the abiotic PCs (independent variables)

Dependent variable	Regression			Independent variable	Coefficient	SE	*t*	*p*	ANOVA	
	r^2	SE	*n*						*F*	*p*
Biotic PC1	0.59	0.71	16	Abiotic PC1	−0.11	0.10	−1.02	0.33	5.79	0.01
				Abiotic PC2	−0.21	0.14	−1.53	0.15		
				Abiotic PC3	0.68	0.16	4.13	0.001		
Biotic PC2	0.33	0.90	16	Abiotic PC1	−0.19	0.13	−1.45	0.17	1.99	0.17
				Abiotic PC2	−0.33	0.18	−1.90	0.08		
				Abiotic PC3	0.30	0.21	1.43	0.18		
Biotic PC3	0.43	1.05	16	Abiotic PC1	0.36	0.15	2.36	0.04	2.95	0.08
				Abiotic PC2	0.03	0.21	0.15	0.89		
				Abiotic PC3	0.27	0.24	1.10	0.29		

contrast between the sediment porewater nutrients and temperature with the sediment redox potential (second abiotic PC), which reflected the influence of the urban effluent.

The higher macroalgae biomass and epiphyte load on *Zostera noltii* leaves found in the meadow closest to the nutrient source (site 1, Fig. 5) reflects the higher availability of nutrients. At high nutrient levels, opportunistic macroalgae and epiphytes are better competitors than seagrasses, since they have higher nutrient uptake and faster growth rates (Duarte, 1995). Thus, they will proliferate and a shift from a seagrass-dominated to a macroalgae-dominated community may occur with increased eutrophication (Sand-Jensen and Borum, 1991; Short et al., 1993; Duarte, 1995; Hauxwell et al., 2003; Cardoso et al., 2004).

Since the loss of seagrass habitats has enormous ecological implications for coastal systems (e.g. decreased productivity and biodiversity, increased sediment resuspension and erosion), and its recovery is likely to be slow or never occur (Duarte, 1995; Hemminga and Duarte, 2000), the conservation of existent seagrass meadows should be imperative to prevent irretrievable losses and, in particular, the effects of the urban wastewater on these communities must be understood and monitored.

We showed here how the nutrient enrichment originated by the urban wastewater discharge affected the population structure, morphology and N content of *Zostera noltii*. The multivariate analysis clearly identified the wastewater discharge as an important source of environmental disturbance and nutrients availability in Ria Formosa lagoon. The high ammonium concentrations (158.3−663.4 μM) at the site closest to the nutrient source showed toxic effects on this species by reducing total biomass and both leaf and internode size. In addition, the higher abundance of macroalgae and epiphytes at this site may also affect the species negatively. The multiple regression analysis showed that two of the main biotic processes operating within *Z. noltii* populations, i.e. the overall size of the plants and the biomass-density dynamics, were significantly correlated to the abiotic processes clearly related to the effects of the urban wastewater, i.e. to the nutrients and anoxia conditions of the sediment, and to the urban wastewater discharge, respectively. The adverse effects of the urban wastewater in *Z. noltii* meadows of Ria Formosa seem to be spatially restricted to areas up to 600 m distant to the WWTW discharge, which is a relatively small spatial impact. The water quality of this coastal system is maintained by a high water renewal as the tidal amplitude is high, up to 3.5 m, and the average depth of the lagoon is low, about 2.5 m.

Acknowledgements

This study was funded by the EC project ‘‘Monitoring and Managing of European Seagrass Beds’’ (EVK3-CT-2000-00044). We are grateful to A. Alexandre, A. Quaresma, C. Alves, C. Godinho, J. Silva, J.A. Bentes and P. Teixeira for fieldwork assistance and to the anonymous reviewers that greatly improved the manuscript. S.C., R.M. and V.V. were supported by PhD grants from Fundação para a Ciência e a Tecnologia (FCT).

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