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Anthropogenic impact on *Zostera noltei* seagrass meadows (NW Iberian peninsula) assessed by carbon and nitrogen stable isotopic signatures

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Keywords

Zostera noltei
N and C stable isotopes
Anthropogenic impact
Temporal evolution

Abstract

Seagrass meadows provide valuable ecosystem services for human wellbeing. They are threatened by increasing human development on coastal areas, which results in eutrophication and ecosystem degradation. The negative effects of anthropogenic pressures on *Zostera noltei* meadows in NW Spain are unknown. This study aims to explore the relationship between watershed human development (i.e., the demographic temporal evolution and land cover indicators of human pressure) and the C and N isotopic signatures determined in *Z. noltei* seagrass meadows located in the related estuarine areas. We measured $\delta^{15}N$, $\delta^{13}C$ and the C:N content of sediment cores, *Z. noltei* leaves and epiphytes collected from three seagrass meadows located at NW Iberian Peninsula characterized by well-differentiated watersheds in terms of the intensity of the anthropogenic pressures (Caldebarcos, Lourizán and A Ramallosa). Ages and sedimentation rates were estimated by $^{210}Pb/^{137}Cs$ dating of one sediment core from the A Ramallosa seagrass meadow, corresponding to the most populated and urbanized watershed. Magnitudes of anthropogenic pressure on the watersheds were determined by the analysis of historic demographic data and the quantification of land cover changes obtained from CORINE Land Cover database. The intense anthropogenic transformation observed in the A Ramallosa watershed resulted in increases of sedimentation rates in the *Z. noltei* meadow. $\delta^{15}N$ and $\delta^{13}C$ signatures revealed the effects of anthropogenic nitrogen inputs. Sediment $\delta^{15}N$ was the variable that best performed as an early warning eutrophication indicator, whereas $\delta^{15}N$ in *Z. noltei* and epiphytic material were less coupled to the magnitudes of artificial land and population density on watersheds.

1. *Introduction*

The human derived alteration of the nitrogen cycle is one of the major processes responsible for eutrophication in coastal areas in general (e.g. Zylicz *et al.*, 1996; Smil, 2001; Gruber and Galloway, 2008; Rockström *et al.*, 2009) and of seagrass meadows, in particular (Duarte *et al*, 2004; Seitzinger *et al.*, 2006; Castro and Freitas, 2011; Mateo, 2015; Sánchez-Lizaso *et al.*, 2015). To address monitoring of this environmental pressure, early warning indicators of anthropogenic nitrogen inputs into aquatic ecosystems, such as stable nitrogen isotopic ratios in sediments and marine biota have been developed (Heaton, 1986; Castro *et al.,* 2007b; Lassauque *et al*., 2010; Kinney and Valiela, 2013; Schubert *et al.*, 2013; Mancinelli and Vizzini, 2015; Roca *et al.*, 2016).

The study of sedimentary records using a combination of $^{210}Pb/^{137}Cs$ dating and the analysis of the $\delta^{13}C$ and $\delta^{15}N$ signatures of organic matter, allows to correlate the historical anthropogenic pressures in the watersheds with anthropogenic nitrogen and carbon inputs (Voss *et al.*,1997, 2000; Zimmerman and Canuel, 2000; Ruiz- Fernández *et al.,*2002; Latimer *et al.*, 2003; Castro *et al.*, 2007a; Gooday *et al.*, 2009; Brandenberger *et al.*, 2011; Ruiz-Fernández *et al.,* 2012; Kinney and Valiela, 2013). The $^{210}Pb/^{137}Cs$ absolute dating radioactive technique is commonly applied to modern sediments as ^{210}Pb can be detected as far as 5 times its semi-disintegration period (22.23 ± 0.12 years) allowing to obtain useful sediment dating spanning 100 years approximately (García-Orellana and Sánchez-Cabeza, 2012). This period comprises the events of demographic and industrial increase in NW Spain watersheds.

Nitrogen inputs into the coastal ecosystem of the Ría de Vigo (NW Spain) largely depend on the wind-driven upwelling system (76% of the total nitrogen entering the Ria), yet the estimated contribution of nitrogen-derived wastewater is also relevant, ca. 22%

(Fernández *et al*., 2016). This contribution is likely to be even more significant in enclosed estuarine areas, where *Zostera noltei* meadows are widely distributed both in intertidal and subtidal zones (Cacabelos *et al*., 2015). However, this fact has not been proved directly, and the effect that these inputs exerted on *Z. noltei* meadows in the region remains largely unknown. In addition, artificial land in coastal areas of the NW Iberian Peninsula increased by 20 % from 1990 to 2000 whereas the human population increased by 13% between 1975 and 1999. Also, land reclamation reduced the coverage of intertidal habitats, lowering their buffer effect against terrestrial organic matter inputs towards estuarine waters (Hernández-Borge, 2002, Díaz *et al*., 2005). It would be, therefore, plausible to hypothesize that inputs of anthropogenic nitrogen and terrestrial carbon towards NW Iberian estuaries have increased in the last decades.

The objective of this study is to explore the response of $\delta^{15}N$ in different compartments of the *Z. noltei* meadows in order to assess their effectiveness as early warning indicators of human derived nitrogen pollution in coastal waters.

For that aim, we measured the $\delta^{15}N$, $\delta^{13}C$, C:N ratios of sediment cores, *Z. noltei* leaves and epiphytes collected from three meadows associated to watersheds differing in terms of human population and intensity of the land artificialization processes. One of the sediment cores sampled in the most impacted *Z. noltei* meadow was also dated using the $^{210}Pb/^{137}Cs$ technique.

2. Materials and methods

2.1. Study areas

The study was conducted in three *Z. noltei* meadows located in estuarine areas of the Northwestern Iberian Peninsula (Spain) (Fig. 1). Study sites were selected on the basis of the, *a priori,* level of anthropogenic impact within the watersheds (Table 1). Caldebarcos was considered the study site with the lowest anthropogenic pressure and was established as the control. The *Z. noltei* meadow located in Caldebarcos covers a small area (0.47 ha) with a patchiness distribution. It is located in the mouth of the small Valdebois river and is protected from the Atlantic Ocean by the Carnota beach. In contrast with the other two *Z. noltei* meadows, Caldebarcos is not inside a ria. A ria is defined as a prolonged inlet located in the shore of NW Spain (see Méndez and Vilas, 2005 for more detail).

The Lourizán study site represented an area with intermediate human pressure, the *Z. noltei* meadow covers a continuous surface of 11 ha. It is located in the southern inner part of the Ría de Pontevedra, near the Lérez river outfall.

The A Ramallosa study site was selected as the zone with the highest anthropogenic pressure. The *Z. noltei* meadow covers 6 ha forming a rather dense canopy and it is located inside an intertidal complex that receives freshwater from the rivers Miñor, Groba and Belesar. The intertidal complex belongs to the municipality of A Ramallosa and is located at the outer southern part of the Ría de Vigo.

Table 1. Summary of anthropogenic impact features in the studied watersheds

Watershed	*Size of the study areas (ha)*	*Inhabitants (2014)*	*Industrial activities*	*Sand mining*	*Recent construction of dams*
Caldebarcos	1588	4376	No	No	No
Lourizán	51671	135989	Large paper factory. Small metal shipyards. Small industrial and commercial surfaces.	Yes (Berducido and Faro sandpits)	Pontillón (1953)
A Ramallosa	9876	43999	Small industrial and commercial surfaces. Machining and metalwork factories. Industrial warehouses.	Yes (Marina sandpit)	Zamáns (1960). Baíña (1985)

2.2. Demographic and land cover analyses

Socio-economic data from the watershed municipalities and changes in land cover of the basins where the municipalities are located were analyzed. Geographical layers of the *Z. noltei* meadows, the rivers, the hydrographic basins and the sampling points were overlapped with the free software tool GvSIG desktop v.2.20. This allowed delimiting the watersheds associated with the studied *Z. noltei* meadows. The watersheds corresponding to Caldebarcos, Lourizán and A Ramallosa covered an area of 1588 ha, 51671 ha and 9876 ha, respectively.

A geographical layer of Spanish municipalities was overlapped over the selected watersheds to select those population entities that occupied the same surface.

In the chosen municipalities, historical demographic statistics such as human population abundance and number of dwelling units were compiled from the Spanish National Statistics Institute (www.ine.es) and from the provincial offices. All available data corresponding to the period between 1842 and 2014 were used. To allow comparison between zones, population abundance and number of dwellings were normalized by the surface of the municipalities.

Watershed-scale land cover changes between 1990 and 2012 were quantified and processed using the CORINE Land Cover (CLC) project data. This data set, which offers land cover classifications for the European Union for 1990, 2000, 2006 and 2012, was downloaded from the Spanish National Geographical Institute database (www.ign.es). The CLC surfaces corresponding to the previously selected watersheds were delimited. For our study, default land cover categories established by the European Environment Agency (EEA) were arranged in new groups of interest for our study, on the basis of the codes corresponding to each land cover (Table 2).

Table 2. Groups used in land cover classification, based on CORINE Land cover codes.

Group	*CLC codes (Level 3)*	*Land cover*
1	1XX	Artificial
2	2XX	Agricultural
3	31X	Forest
4	32X, 333	Shrub and sparse vegetation
5	332, 334	Burnt area and bare rock
6	42X, 331	Coastal wetlands and beaches
7	521, 522	Coastal lagoons and estuaries
8	51X, 41X	Inland waters

We noticed that in the watershed of Lourizán, areas formerly classified as artificial land in 1990 turned to be natural land in years 2000, 2006 and/or 2012. This unusual observation led us to revise and correct land cover classification in our studied watersheds. Actual and former ortho-photography was used to verify that polygons actually represented the category given. If not, polygons were re-codified or re-drawn according to the methodological guides published by the EEA (http://www.eea.europa.eu/). This kind of errors in the CLC land cover classification have been detected in other studies (Catalá-Mateo *et al.*, 2008; Barreira-González *et al.*, 2012). Once new groups were defined, the relative contribution of each land cover class was calculated. Then, differences for the study period were calculated by subtracting absolute areas. Relative magnitudes of the changes per each category were calculated following expression (1),

(1) $$\Delta_{relative}(\%)=\frac{(\text{area}_{t=n+1}\text{ - area}_{t=n})}{\text{area}_{t=n}}\cdot 100$$

where $\text{area}_{t=n}$ means the coverage at a specific year, and $\text{area}_{t=n+1}$, means the coverage in the following year when data were available.

2.3. Sample collection

Samples were collected at low spring tides, when the *Z. noltei* meadows emerged, in April 2014 at A Ramallosa, September 2014 at Caldebarcos and December 2014 at Lourizán. Since the *Z. noltei* samples were collected at different seasons, the biological fractionation exerted by the plant and the epiphytes caused by seasonality can alter the isotopic ratios and complicate a straightforward interpretation of the observed isotopic signatures on the basis of anthropogenic nitrogen inputs (Román *et al*., 2018).

At each sampling site, sediment cores were collected by manually pushing 9 cm diameter PVC cylinders into the sediments. A total of 7 cores were sampled: 3 cores (A, B and C) at A Ramallosa, 2 cores (D and E) at Caldebarcos, and 2 cores (F and G) at Lourizán (Fig. 1). Sediment cores were stored refrigerated, cut longitudinally and, one of the longitudinal cuts separated in 1 cm transversal sections. The sediment subsamples, corresponding to each transversal section, were dried in an oven at 60 ºC until they reached constant weight and stored for further analysis.

Samples of *Z. noltei* leaves were also taken from each seagrass meadow by cutting random leaves from the above-ground biomass. We established 4 sampling points in Caldebarcos, 5 in Lourizán and 3 in A Ramallosa (Fig. 1). At each of the points in Lourizán and A Ramallosa, three replicates of *Z. noltei* leaves were taken, obtaining a total of 15 and 9 samples respectively. In Caldebarcos replicates were not sampled due to the very small surface covered by the *Z. noltei* meadow. *Z. noltei* leaves were placed in plastic bags and stored refrigerated until immediate analysis.

2.4. Isotopic analysis

Sediment subsamples were grounded with a ball mill equipped with zirconium oxide grinding jars until achieving a particle size lower than 100 µm. Grinded samples were stored in a closed container and acidified with 37 % HCl vapor during 24 hours in order to remove carbonates. When sediment samples showed $\delta^{13}C$ ratios indicating a likely presence of particulate inorganic carbon ($\delta^{13}C$ between -1 to -20 ‰), they we re-acidified for 48 hours until reaching a total acidification time of 72 hours and they were analyzed again. When $\delta^{13}C$ ratios were under the cited threshold we assumed that carbonates were completely removed from the samples. Then, samples were dried at 60ºC for 48 h.

Z. noltei leaves were first rinsed with water to remove mud and detritus. Then, the epiphytic material was extracted by scrapping the leaves with a glass plate. Then, leaves were cleaned with distilled water and the water containing the epiphytes was filtered through a 100 µm mesh. Both the leaves and the epiphytic material were separately dried in an oven at 60 ºC until constant weight. As with the sediment subsamples, once *Z. noltei* leaves were dry, they were grounded in a ball mill until achieving a particle size lower than 100 µm.

Z. noltei leaves were not acidified. Previous acidification experiments carried out with samples from A Ramallosa (Román *et al*., 2018), showed that $\delta^{13}C$ ratios did not show significant differences between acidified ($\delta^{13}C$ = -14.0 ± 0.5 ‰) and non-acidified ($\delta^{13}C$ = -14.0 ± 0.7 ‰) *Z. noltei* samples (p>0.05, n= 5), suggesting that the amount of carbonates attached to these leaves are likely to be negligible.

C:N ratios and the $\delta^{13}C$ and $\delta^{15}N$ isotopic signatures of sediments, *Z. noltei* leaves and epiphytes were analyzed by Isotopic Ratio Mass Spectrometry (IRMS) at the research support service (SAI) of the University of A Coruña, using an elemental analyzer FlashEA1112 coupled with an Interface Conflo II (ThermoFinnigan) to an isotopic ratio mass spectrometer Deltaplus (ThermoFinnigan). In each analytical sequence secondary standards used for $\delta^{15}N$ were IAEA-N-1 (+0.4‰), IAEA-N-2 (+20.3‰) and USGS-25 (-30.4‰). For $\delta^{13}C$, the secondary standards were NBS 22 (-30.031‰), IAEA-CH-6 (-10.449‰) and USGS 24 (-16.049‰). To assess the precision (standard deviation), acetanilide ($\delta^{15}N$ = -8.47 ‰, $\delta^{13}C$ =-25.09 ‰) was used as a standard, with a resulting SD of ± 0.15 ‰ (n=10) both for $\delta^{13}C$ as for $\delta^{15}N$. $\delta^{13}C$ and $\delta^{15}N$ results were expressed in per mill (‰) notation relative to the international standards VPDB (Vienna Pee Dee Belemnite) and atmospheric air (Sulzman, 2007). The presence of numerous bivalve shell fragments in the Caldebarcos study site prevented a complete removal of inorganic carbonates through the acidification treatment performed, as shown in $\delta^{13}C$ results which suggest the presence of inorganic carbon. Consequently, the C:N and $\delta^{13}C$ results from this study site were not used.

2.5. $^{210}Pb/^{137}Cs$ dating

The remaining longitudinal half of core A (A Ramallosa) was subsampled in 45 sections with a thickness of 1 cm for $^{210}Pb/^{137}Cs$ dating. The $^{210}Pb/^{137}Cs$ dating technique is based on the ^{210}Pb in excess ($^{210}Pb_{xs}$) measured, with respect to the ^{210}Pb coming from the disintegration of ^{226}Ra existing in the sedimentary matrix ($^{210}Pb_{eq}$) (Sánchez-Cabeza and Ruiz-Fernández, 2012). $^{210}Pb_{eq}$ values are obtained from the determination of ^{226}Ra, and the $^{210}Pb_{xs}$ activity is estimated as the difference between $^{210}Pb_{total}$ and $^{210}Pb_{eq}$ (Rizzo *et al.,* 2009).

Each subsample corresponding to 1 cm of depth was grounded in a ball mill, placed in ethanol-washed methacrylate cylindrical boxes and sealed with cyanoacrilate. Samples were analyzed in the Ionizing Radiation laboratory at the University of Salamanca. For each of the subsamples, ^{210}Pb, ^{226}Ra and ^{137}Cs activities were simultaneously determined by low-level background gamma-ray spectrometry in a hyper pure germanium detector "n" type, with a relative efficiency of 30%. Obtained spectrums were analyzed by the laboratory staff with the gamma-line analyzer software tool "Galea" (Quintana *et al.*, 2006). The Constant Rate of Supply (CRS) model was applied to the core. The CRS model assumes a constant flux of $^{210}Pb_{xs}$ to the sediments, and takes into account the sediment compaction by previously knowing the mass thickness of each depth (Geometric thickness · density, expressed in g · cm^2). The model allows determining the ages for each sampling interval, and consequently, the sedimentation rates (Carrol and Lerche, 2003). To ascertain that obtained ages were correct, the ^{137}Cs profile was assessed by checking the ^{137}Cs maximums. Depth and age values corresponding to the ^{137}Cs maxima should coincide with the atmospheric nuclear tests that began in the decade of the 40's, and whose maximum intensity corresponded to 1963 (Alonso *et al.*, 2015). Also, ^{137}Cs maxima should match with the Chernobyl accident in 1986 and the Fukushima accident in 2011.

2.6. Data treatment

Linear regression analyses between $\delta^{15}N$ and depth were performed on the data derived from the sediment cores collected.

Differences between the three study zones in $\delta^{15}N$ values from surface sediments, *Z. noltei* leaves and epiphytes and from sediment cores averaged per 0-49 cm depth, were assessed by performing one-way ANOVAs and post hoc Tukey tests. Replicates of *Z.*

noltei leaves and epiphyte samples were pooled for the mean comparison tests, thus obtaining n=4 in Caldebarcos, n= 15 in Lourizán and n= 9 in A Ramallosa. Surface sediments were defined as those located between 0 and 5 cm, as 5 cm was the highest depth reached by the *Z. noltei* below ground biomass. Number of samples of surface sediment used for the mean comparison test were n=10 in Caldebarcos, n=10 in Lourizán and n=15 in A Ramallosa. Normality and homoscedasticity of the variables were checked. Non-parametric tests (Kruskall-Wallis) were carried out when data transformations were not able to make data obey the normality and homoscedasticity assumptions.

2.7. Mass balance estimations

The relative contribution of nitrogen and carbon sources to the sediment in Lourizán and A Ramallosa were estimated from isotopic mixing models using the Iso source 1.3.1 software (Phillips and Gregg, 2003).

We estimated the approximate relative contribution (F) of nitrogen from sewage, natural (non-anthropogenic) origin and N_2 fixation to the total nitrogen inputs in the estuaries using the $\delta^{15}N$ signature of surface sediments. Similarly, the relative contributions of matter from *Z. noltei*, terrestrial and marine sources to the total inputs were estimated from $\delta^{13}C$.

The end-members (isotopic signatures that are representative of particular sources of organic matter) used were chosen from the literature and also from the background results obtained in this study (i.e, the low population density and the lack of conspicuous sewage matter outfalls in the Caldebarcos zone allowed us to assume that N from sewage origin in this site was likely to be negligible). We therefore used the average $\delta^{15}N$ value measured in the first 5 cm of the sediment cores (4.6 ‰) sampled at Caldebarcos as the natural end-member (see section 3.3). The characteristic sewage end-member for sediments (8.5 ‰) was obtained from Xiao and Liu (2010), who analyzed $\delta^{15}N$ values in the sediment at a sewage discharge point. However, we are aware that the actual $\delta^{15}N$ sewage end-member may be significantly different from those found in the literature. The N_2 fixation end-member was 0 ‰, since this process generates an almost negligible alteration of the original $\delta^{15}N$ of the atmospheric nitrogen (Heaton, 1986; Kendall *et al*., 2007; Michalski *et al*., 2015).

Z. noltei end-members were extracted from the average $\delta^{13}C$ values in *Z. noltei* from A Ramallosa (-12.0 ‰), and from Lourizán (-14.6 ‰) obtained in this study. The selected sediment $\delta^{13}C$ marine end-member was -20 ‰ (Libes, 1993) and the terrestrial end member was -26 ‰ (Latimer *et al.*, 2003).

We are aware of the limitation associated to the use of end-member values from the literature. Consequently, the results of these estimations should be taken with caution.

3. Results

3.1. Human population data and land cover

Human population (termed "population" hereafter) and the density of dwelling units dwelling densities in the three watersheds followed the anthropogenic pressure gradient established *a priori* and were representative of contrasting levels of human pressure (Fig 2). In the 1845-2014 period population density increased by 0.08 % $\cdot year^{-1}$ in Caldebarcos, and 1 % $\cdot$ $year^{-1}$ in Lourizán and A Ramallosa. In the same temporal period, the density of dwelling units increased by 0.5%$\cdot year^{-1}$ in Caldebarcos, 4.3% $\cdot year^{-1}$ in Lourizán and 3% $\cdot year^{-1}$ in A Ramallosa. In 1960, population and dwelling densities were almost equal in both Lourizán and A Ramallosa, yet then these densities increased more intensively in the latter.

This gradient in demographic pressure agreed with the evolution of artificial surfaces at the study sites, as the relative increase in artificial land class for the 1990- 2012 period was negligible in Caldebarcos, intermediate in Lourizán and the highest in A Ramallosa (Table 3).

Table 3. Relative change of the areas (%; ha_{2012} - ha_{1990}) / ha_{1990}) of each land cover category during the studied period (1990-2012). The area (ha) covered by each class in 2012 is indicated in brackets, next to the relative change area. “n.a.” indicates that relative changes of artificial land could not be calculated in Caldebarcos, due to absence of artificial land cover in 1990. “i” indicates inexistence of the land cover type.

Site	*Artificial Land (%)*	*Agricultural areas (%)*	*Forest (%)*	*Shrub and sparse vegetation (%)*	*Burnt area (%)*
Caldebarcos	n.a. (0.9174)	-33 (193)	-35 (225)	26 (1039)	-100 (0)
Lourizán	50 (2073)	-15 (10548)	49 (22487)	-27 (16267)	-100 (0)
A Ramallosa	265 (918)	-25 (2241)	3 (5362)	-7 (1228)	i

3.2. Short term responses: $\delta^{13}C$, $\delta^{15}N$ and C:N ratio in surface sediments, Z. noltei and epiphytes

The $\delta^{15}N$ in surface sediments showed differences between the three seagrass meadows, being significantly higher in A Ramallosa than in Lourizán, and in Lourizán than in Caldebarcos. Thus, $\delta^{15}N$ in surface sediment showed a coupled increase with the degree of anthropogenic pressure in the watersheds.

$\delta^{15}N$ ratios in *Z. noltei* and epiphytes were less coupled to the degree of anthropogenic pressure than in the case of surface sediments. $\delta^{15}N$ values in *Z. noltei* leaves were significantly higher in A Ramallosa than in Lourizán, but $\delta^{15}N$ values at Caldebarcos were not significantly different from those measured in the other two meadows. Besides, in Lourizán, atypically low $\delta^{15}N$ values were measured and the mean relative nitrogen content (%) in leaves from Lourizán (3.7 ± 0.3 %) was significantly higher ($p<0.05$) than the values measured in A Ramallosa (3.4 ± 0.2 %) and Caldebarcos (2.5 ± 0.2 %). As for *Z. noltei,* $\delta^{15}N$ in epiphytes were significantly lower in Lourizán than at the other two meadows (Fig. 3).

3.3. *$\delta^{15}N$, $\delta^{13}C$ and C:N ratio in sediment cores*

Raw profiles of $\delta^{15}N$ and C:N ratios suggested that the most impacted meadow (A Ramallosa) received higher inputs of anthropogenic nitrogen than Lourizán and Caldebarcos, and that these inputs were increasing towards recent layers (Figs. 4 and 5).

The averaged $\delta^{15}N$ along the sedimentary records was significantly higher in A Ramallosa (5.2 ± 0.6 ‰) than in Lourizán (4.8 ± 0.7 ‰; $p = 1.34 \cdot 10^{-6}$), and in Caldebarcos (4.7 ± 0.6 ‰; $p = 1 \cdot 10^{-6}$). Also, the C:N ratio in A Ramallosa (12 ± 4) was significantly lower ($p = 2.7 \cdot 10^{-15}$) than in Lourizán (12 ± 2), indicating higher relative inputs of anthropogenic N in the most impacted meadow (Fig. 6).

Moreover, linear regressions performed with $\delta^{15}N$ ratios (Y) and depth (X) along the entire cores, confirmed that $\delta^{15}N$ increased towards the surface in A Ramallosa, whereas in Lourizán and in Caldebarcos, $\delta^{15}N$ decreased towards surface, or remained constant (Core E) (Table 4)

Table 4. Linear regression models between $\delta^{15}N$ in sediment, $\delta^{15}N$ (y) and depth (x) for all cores sampled in Lourizán and A Ramallosa. Study sites are arranged following increasing population density and artificial land cover in the related watersheds. Significance level: $p < 0.05$

Site	*Core*	*Regression equation*	R^2	*p value*
Caldebarcos	D	$\delta^{15}N = 4.4 + 0.02 \cdot$ depth	0.3	$6.5 \cdot 10^{-6}$
Caldebarcos	E	-	-	0.07 (n.s.)
Lourizán	F	$\delta^{15}N = 4.3 + 0.009 \cdot$ depth	0.09	$9.8 \cdot 10^{-3}$
Lourizán	G	$\delta^{15}N = 4.7 + 0.02 \cdot$ depth	0.2	$1.2 \cdot 10^{-4}$
A Ramallosa	A	$\delta^{15}N = 6.4 – 0.03 \cdot$ depth	0.7	$2.2 \cdot 10^{-16}$
A Ramallosa	B	$\delta^{15}N = 5.1 – 0.01 \cdot$ depth	0.07	0.03
A Ramallosa	C	$\delta^{15}N = 5.6 – 0.01 \cdot$ depth	0.7	$2.2 \cdot 10^{-14}$

The dated core (core A from A Ramallosa), allowed us to (i) estimate sedimentation rates and (ii) correlate isotopic and C:N values with the anthropogenic impact of the A Ramallosa watershed. In the studied core, $^{210}Pb_{ex}$ was not detectable below 38 cm depth. The oldest reliable age obtained was 1892 ± 23 (122 ± 23 years before sampling) at 30 cm depth. In the upper 10 cm sediment layer, the $^{210}Pb_{total}$ profile showed a relatively constant trend and an exponentially decaying trend with depth was not observed (data not shown), suggesting the existence of a mixed sedimentary layer (Fig. 7) likely caused by hydrodynamics and bioturbation (Middelburg *et al.*, 2004; Jankowska *et al.*, 2016).

The upper 15 cm layer, where the most marked changes in $\delta^{15}N$, $\delta^{13}C$ and in the C:N ratio occurred, corresponded with the period 1970-2014. In this period, population and density of dwelling units increased exponentially (Fig 2).

Increases in sedimentation rates correlated positively with the $\delta^{15}N$ isotopic enrichment in A Ramallosa cores (Pearson correlation coefficient r = 0.6510, *p<0.001*), indicating that higher sediment rates in the meadow were associated with a higher ^{15}N enrichment in the sediments.

Simple linear regressions performed between $\delta^{15}N$ and $\delta^{13}C$ of the dated core "A" from A Ramallosa, and population and densities of dwelling units at the watershed corresponding to the same age of the isotopic ratios, showed statistically significant positive relationships between population density in the most impacted watershed (A Ramallosa) and the corresponding $\delta^{15}N$ ($p < 0.0001$) and $\delta^{13}C$ ($p < 0.05$) sedimentary values. Also, $\delta^{15}N$ increased significantly ($p < 0.0001$) with the increase in dwellings density (Fig. 8).

4. Discussion

In the coastal areas under analysis, we corroborated that *Z. noltei* and its epiphytes are not a useful indicator of nutrient enrichment as compared with the response observed in the sediments. *Z. noltei* and epiphytes selectively assimilate the lighter isotopes under non-limiting conditions and their nutrient requirements vary as a function of the season (Marshall *et al.*,2007; Roca *et al.,* 2016). These isotopic fractionation processes modulate the C and N isotopic signatures, and limit the straightforward interpretation of $\delta^{15}N$ in *Z. noltei* meadows as nutrient enrichment indicators.

Accordingly, $\delta^{15}N$ in *Z. noltei* did not respond directly to increases in anthropogenic pressure. Values were significantly higher in A Ramallosa than in Caldebarcos, consistent with the relatively undisturbed nature of this area. In Lourizán, by contrast, $\delta^{15}N$ values were among the lowest ever reported (Table 5) both in *Z. noltei* leaves and in epiphytes. This observation was not expected a priori, given the intermediate anthropogenic pressure characteristic of this watershed.

The very low $\delta^{15}N$ values measured in *Z. noltei* in Lourizán were probably related to the assimilation of ammonium by the plants in this NH_4^+-rich area. The ammonium released from organic N is an important source of isotopically depleted nitrogen (Heaton, 1986; Möbius, 2013) and *Z. noltei* has shown thirty times more affinity for ammonium than for nitrate (Alexandre *et al*., 2011; Walton *et al.* 2016). The *Z. noltei* meadow of Lourizán is in the inner part of the Ría de Pontevedra (Fig. 1), whereas the other two meadows, A Ramallosa and Caldebarcos, are located in the outer Ría de Vigo and exposed to the Atlantic Ocean, respectively. Previous studies have reported high ammonium concentrations in the inner part of the Ría de Pontevedra where Lourizán is located (Doval *et al.,* 2016). For instance, Doval *et al*. (2016), during a 9-year study, found that in the inner stations of the Galician rias (Vigo, Pontevedra, Arousa, Muros and Ares-Betanzos) average ammonium concentrations were 140% higher than in outer stations.

Table 5. $\delta^{15}N$ mean and standard deviations from *Z. noltei* leaves at meadows located in the European Atlantic coast.

Location	*$\delta^{15}N$ (‰)* *Z. noltei leaves*	*Season*	*Reference*
Lourizán. Pontevedra ria. Spain	3 ± 2	Autumn	This study
Inland sea. United Kingdom	3.3 ± 0.6	Spring	Papadimitriou *et al.* (2006)

Mira estuary. Portugal	3 ± 2	Summer and winter	Vafeiadou *et al.* (2014)
Arcachon Bay. France	4 ± 1	Spring	Dang *et al.* (2009)
Ria Formosa. Portugal	4.5	Summer	Machás and Santos (1999)
Mira estuary. Portugal	4.9 ± 0.5	n.a.	Castro *et al.* (2007a)
Arcachon Bay. France	5.5 ± 1.4	Spring	Dubois *et al.* (2012)
Northern Galician rias. Spain	5.6	Summer	Bode *et al.* (2006)
Caldebarcos. NW Galicia. Spain	5.8 ± 0.3	Autumn	This study
Inland sea. United Kingdom	7.5	Summer	Papadimitriou *et al.* (2006)
A Ramallosa. Vigo ria. Spain	7.9 ± 0.5	Spring	This study
Mondego estuary. Portugal	8.2 ± 0.3	n.a.	Castro *et al.* (2007a)
Mondego estuary. Portugal	8.9 ± 0.4	Summer	Rossi *et al.* (2015)
Mondego estuary. Portugal	9.5 ± 1.5	All seasons	Baeta *et al.* (2009)
Arcachon Bay. France	9.9 ± 0.8	Autumn	Lebreton *et al.* (2012)

The epiphytes from the Lourizán seagrass meadow also showed the lowest $\delta^{15}N$ values. Epiphytes from various seagrass species (*Thalassia testudinum, Posidonia oceanica* and *Cymodocea nodosa*) have shown different assimilation preferences of either NH_4^+ or NO_3^- (Cornelisen and Thomas, 2002, 2006; Lepoint *et al.*, 2004, 2007; Apostolaki *et al.*,2012; Van Engeland *et al.*, 2013). This lack of agreement can be caused by the variability in the relative composition of heterotrophs vs. autotrophs (Hemminga and Duarte, 2000; Lassauque *et al.*, 2010). In this study we did not investigate the composition of epiphytes, yet the low $\delta^{15}N$ in the Lourizán epiphytes could result from the preferential uptake for NH_4^+. Further ^{15}N tracer experiments in *Z. noltei* meadows could help to clarify which nutrient source if preferred by their epiphytes. Overall, results evidence the poor efficacy of $\delta^{15}N$ in epiphytes as direct indicators of anthropogenic nutrient inputs.

By contrast, the $\delta^{15}N$ ratios in sediment cores, jointly with demographic and land cover changes from the watersheds offered a clearer and more complete view of the human derived nitrogen enrichment in the study sites.

In sediment records of Caldebarcos and Lourizán, $\delta^{15}N$ did not show a general increasing trend towards the surface. These patterns indicated lower recent anthropogenic nitrogen inputs to the meadows and were consistent with the lower artificial surfaces, and lower increases of population and dwelling units in the watersheds. By contrast, $\delta^{15}N$ increased towards recent layers in A Ramallosa, what is consistent with the increase of anthropogenic pressure indicators in the watershed during the most recent decades. The increase in human population and land artificialization in the A Ramallosa watershed since the middle of the 20th century are likely to be associated with increases in sediment inputs and anthropogenic nitrogen loadings into the seagrass meadow. The coastal area surrounding the A Ramallosa salt marsh experienced a significant increase in anthropogenic pressure over the last five decades (Fig. 2). In 2001, the fraction of secondary residences with respect to total residences exceeded 30 %; corresponding to one of the highest population growth rates measured in NW Spain (Hernández-Borge, 2007). The increase of sedimentation rates reflected the rise in erosion and runoff produced by human induced changes in land cover (Ruiz-Fernández *et al*., 2002; Macreadie *et al*., 2012).

The most important carbon inputs both in Lourizán and A Ramallosa were from terrestrial origin (Table 5). These contributions of terrestrial material were probably caused by important fluxes of sediments from the river outfalls that are characteristic of estuarine environments (Evans *et al*., 2011). Yet in Lourizán the most abundant N source was of natural origin, whereas in A Ramallosa 51 ± 11 % of the N derive from sewage sources

(Table 5). Estimates of the nitrogen supply to the Ría de Vigo have indicated that 22% of the nitrogen inputs were from sewage origin (Fernández *et al.*, 2016). So our higher estimates are likely a consequence of the location of the study site: an intertidal complex that is protected by a vegetated littoral sand-bar, which creates a low-energy hydro dynamism (Alejo *et al*., 1990) and enhances sediment preservation (Méndez-Martínez *et al.,* 2011). Nevertheless, the lack of local $\delta^{13}C$ and $\delta^{15}N$ end members is a methodological limitation that should be considered to interpret the reliability of the results derived from the mass balance estimations.

Table 5. Relative contributions of the main carbon and nitrogen sources towards the surface sediments in Lourizán (Intermediate anthropogenic pressure) and A Ramallosa (Higher anthropogenic pressure).

Sites	*Carbon sources (%)*			*Nitrogen sources (%)*		
	Z. noltei	*Marine*	*Terrestrial*	*N_2 fixation*	*Natural*	*Sewage*
Lourizán	10 ± 6	23 ± 14	68 ± 8	21 ± 12	46 ± 27	32 ± 15
A Ramallosa	5 ± 3	12 ± 7	83 ± 4	15 ± 9	34 ± 20	51 ± 11

Large fluxes related to discharges from sewage outfalls have been found to be associated with sediments enriched in $\delta^{15}N$ and $\delta^{13}C$, and low C:N ratios (Ruiz-Fernández *et al.* 2002; Michener and Kaufman, 2007, Xiao and Liu, 2010). The increasing nitrogen inputs into the meadows lead to a fertilization of the estuarine water and enhancement of phytoplankton production, generating organic matter with higher $\delta^{13}C$ than the organic matter from terrestrial origin (Craig, 1953; Libes, 1993; Gooday *et al.*, 2009). The relatively low measured C:N ratios from recent layers, characteristic of macroalgae and phytoplankton (5-15) (Finlay and Kendall, 2007) confirmed an increasing contribution of marine-derived organic matter.

Global anthropogenic factors, such as global warming and the subsequent sea level rise, can influence the distribution patterns of $\delta^{15}N$ and $\delta^{13}C$ signatures in recent sediment layers. $\delta^{15}N$ in the sedimentary record can vary if the balance of marine vs. riverine DIN inputs varies, since in the region, $\delta^{15}N$ in DIN from upwelling nitrate is usually higher (5 - 6 ‰) (Marconi *et al*., 2014) than riverine DIN (~ 4 ‰) (Bode *et al*., 2011). The first 15 cm of the sediment in A Ramallosa, which corresponded to the period 1970-2014, showed the highest increases of $\delta^{15}N$. Sea-level rise estimates for the Ría of Vigo yield values of 2.68 mm per year (Rosón *et al.*, 2009), what would result in an increase of nearly 11.8 cm in the 1970-2014 period. An increase in sea level would result in a higher relative contribution of marine matter, and therefore, an increase in sedimentary $\delta^{15}N$ and $\delta^{13}C$ values. So the observed increase of $\delta^{15}N$ and $\delta^{13}C$ in the upper sedimentary layers can be partially explained by the effect of sea level rise characteristic of recent decades.

The rise in sedimentary $\delta^{15}N$ associated with increases in human population in the watershed of A Ramallosa is in agreement with previous results reported for various estuaries, such as the Baltic sea (Voss *et al.*, 2000), Chesapeake Bay in USA (Zimmerman and Canuel; 2000), New Bedford Harbor in USA (Latimer *et al*., 2003), the Mondego estuary in Portugal (Castro *et al*., 2007b), or the densely populated Jamaica Bay in USA (Wigand *et al*., 2014). The worldwide reported effect of anthropogenic pressure on estuarine $\delta^{15}N$ has here been shown for the first time in *Z. noltei* meadows at NW Spain.

In conclusion, the results found in this study showed a pronounced rise of anthropogenic pressure, increasing sedimentation rates and evidences of human derived nitrogen enrichment in the A Ramallosa watershed. Sedimentary $\delta^{15}N$ responded directly to changes in anthropogenic pressure, whereas $\delta^{15}N$ in *Z. noltei* and epiphytic material were not directly related to the degree of human pressure, indicating that the isotopic signature of these organisms are not feasible indicators of the degree of anthropogenic nitrogen enrichment in estuarine ecosystems.

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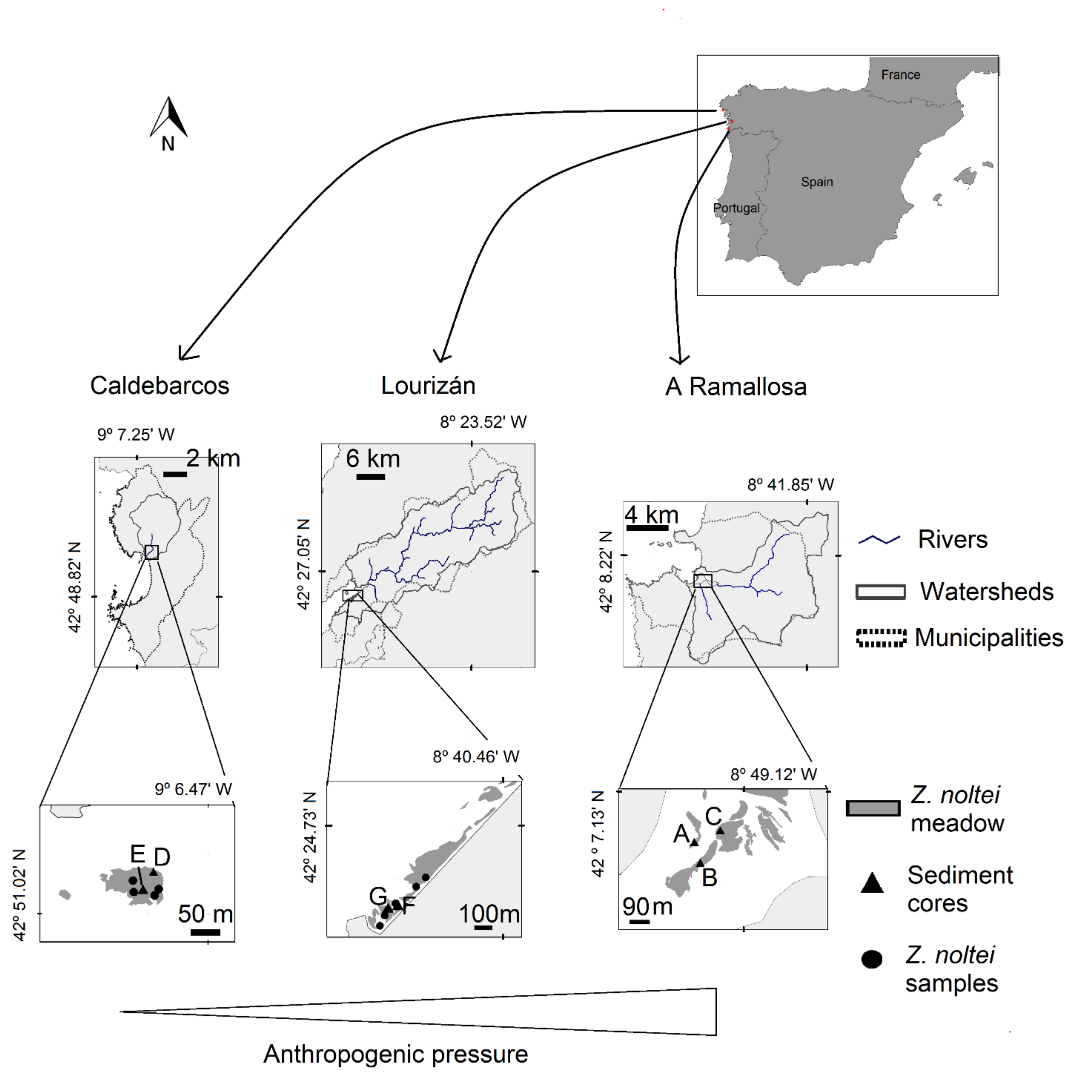

Fig. 1 Study areas and sampling stations where sediment corers and *Z. noltei* plants were collected. Watersheds and municipalities indicate areas where land cover and demographic analyses were carried out, respectively. Cores were named from A to G. In A Ramallosa, sediment cores and *Z. noltei* samples were collected at the same locations

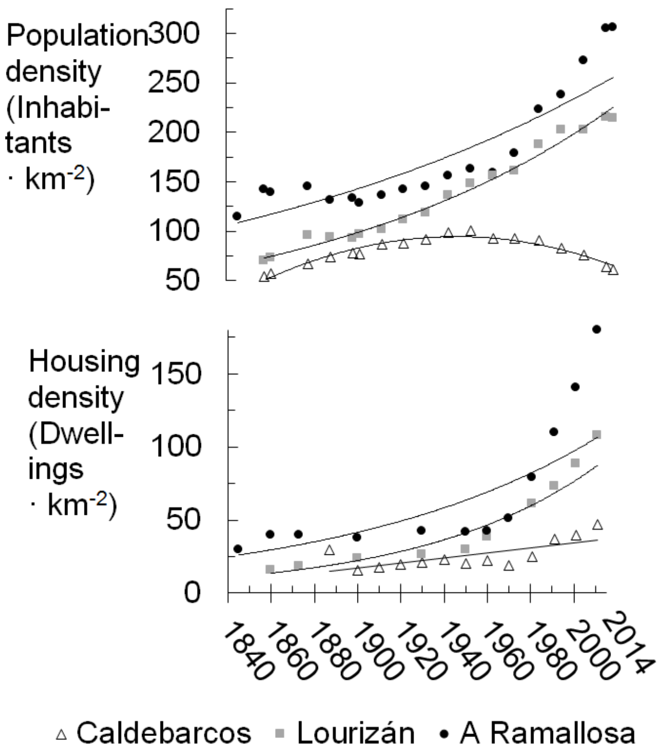

Fig. 2 Temporal evolution (1850-2014) of human population and dwellings densities in Caldebarcos (triangles), Lourizán (squares) and A Ramallosa (circles)

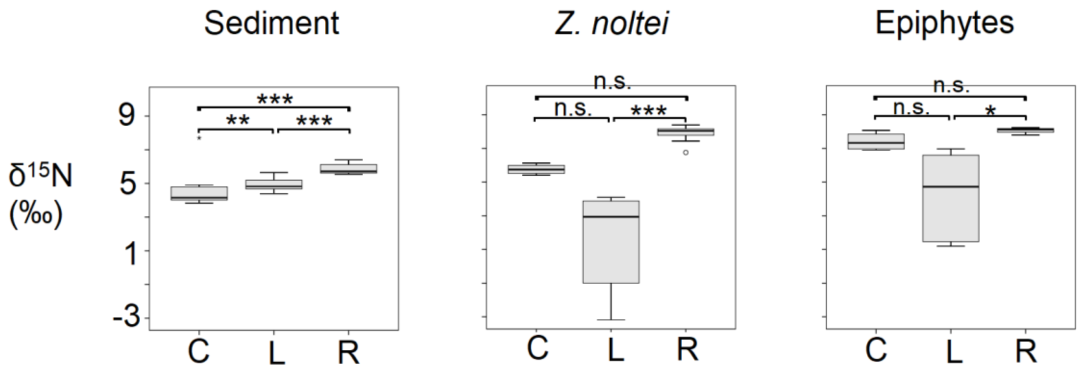

Fig. 3 Box plots of $\delta^{15}N$ measured in surface sediment (n=35) (from 0 to 4 cm), *Z. noltei* leaves (n=28) and epiphytes (n=28) at the seagrass meadows of Caldebarcos (C), Lourizán (L) and A Ramallosa(R). Study sites were arranged according to increasing human population and artificial land coverage in the watersheds. Asterisks indicate significant differences between sites (***= p< 0.001, ** = p < 0.01 , * = p < 0 .05), "n.s." = not significant differences.

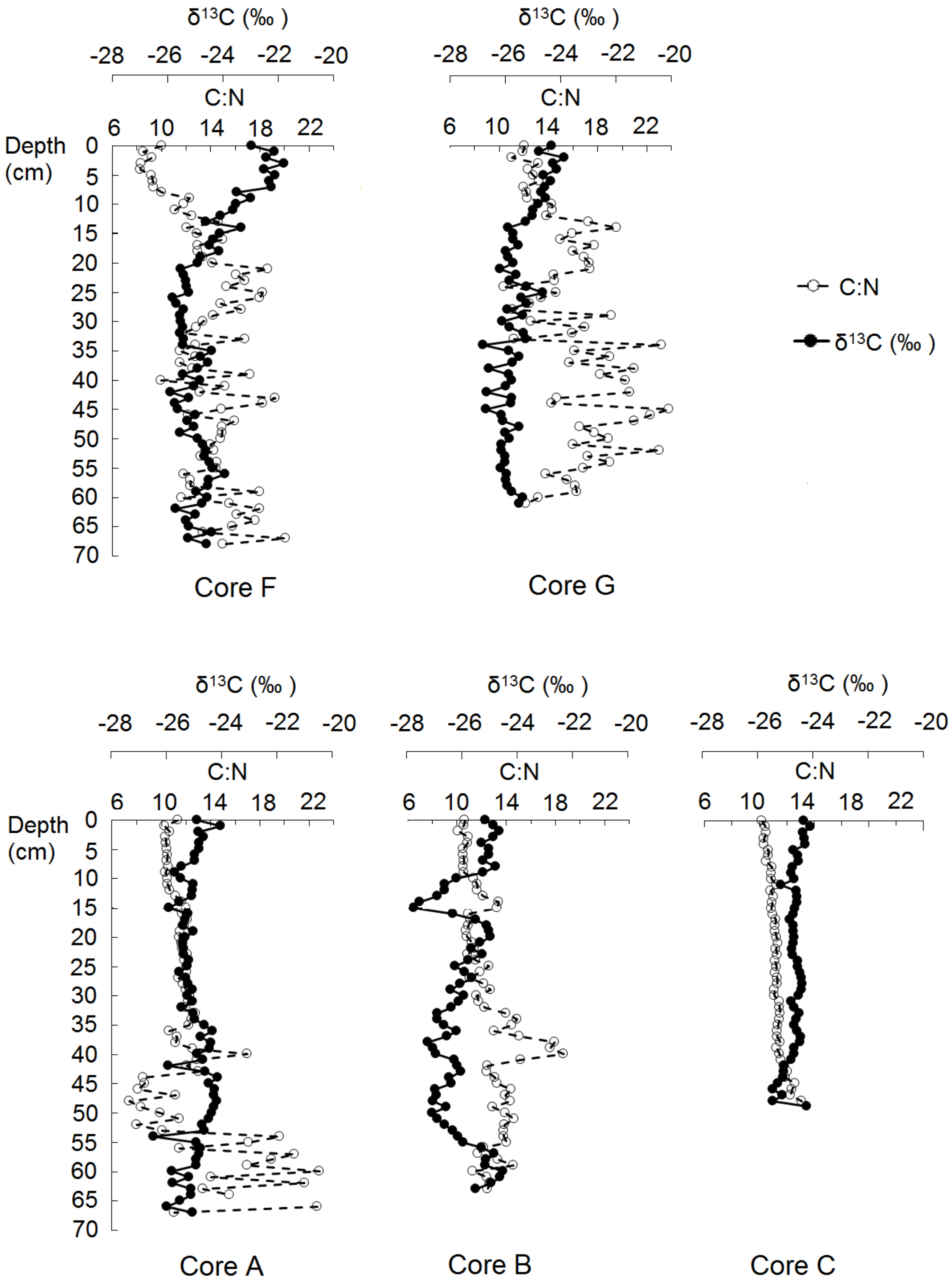

Fig. 4 C:N ratio and $\delta^{13}C$ sediment cores sampling in Lourizán (core F and G; Intermediate anthropogenic pressure) and in A Ramallosa (cores A, B and C; High anthropogenic pressure).

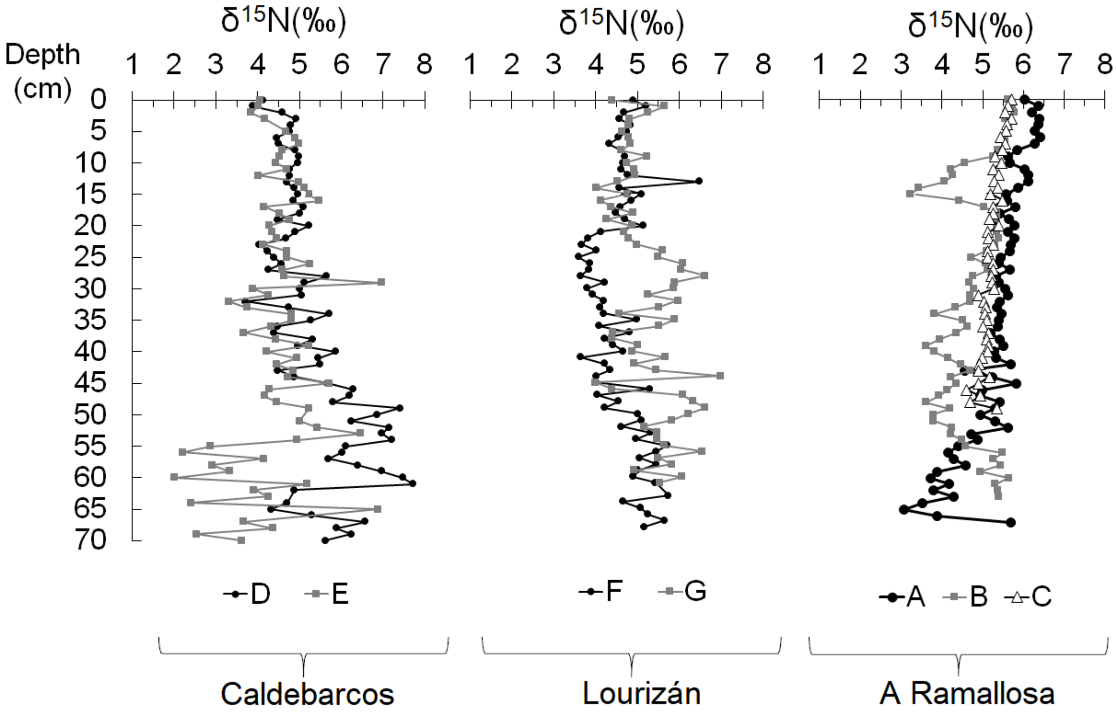

Fig. 5 $\delta^{15}N$ depth profiles in sediment cores from the three *Z. noltei* meadows studied. Sediment profiles are arranged according to increasing population densities and artificial land cover in the sampled watersheds.

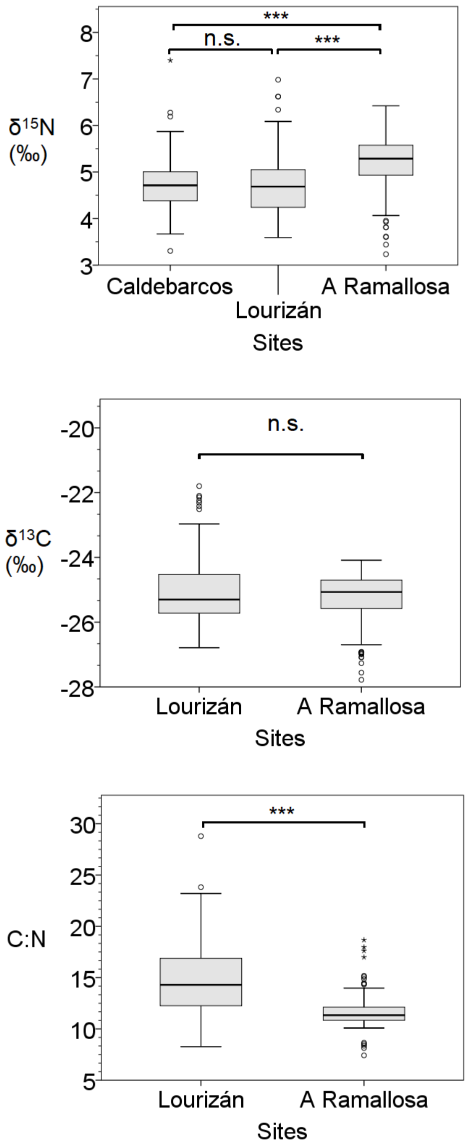

Fig 6. Box plots of $\delta^{15}N$, $\delta^{13}C$ and C:N averaged per depth (0 to 49 cm) for the cores at each study site. Asterisks indicate significant differences between sites (***= p< 0.001), "n.s." = not significant differences.

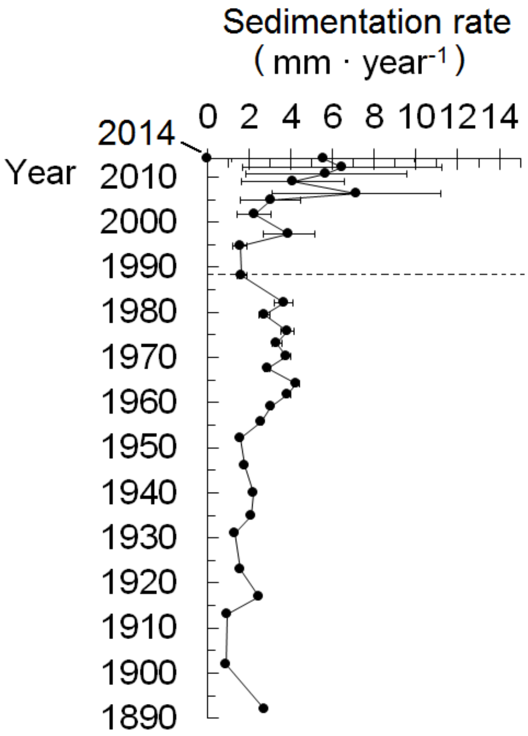

Fig. 7 Sedimentation rates and ages obtained by $^{210}Pb/^{137}Cs$ dating in the first 30 cm in core "A" sampled at A Ramallosa *Z. noltei* meadow (Coordinates: 42º 7.013'N, 8º 49.272' W). Error bars denote the standard deviations. Discontinuous line indicates the limit of the mixed layer

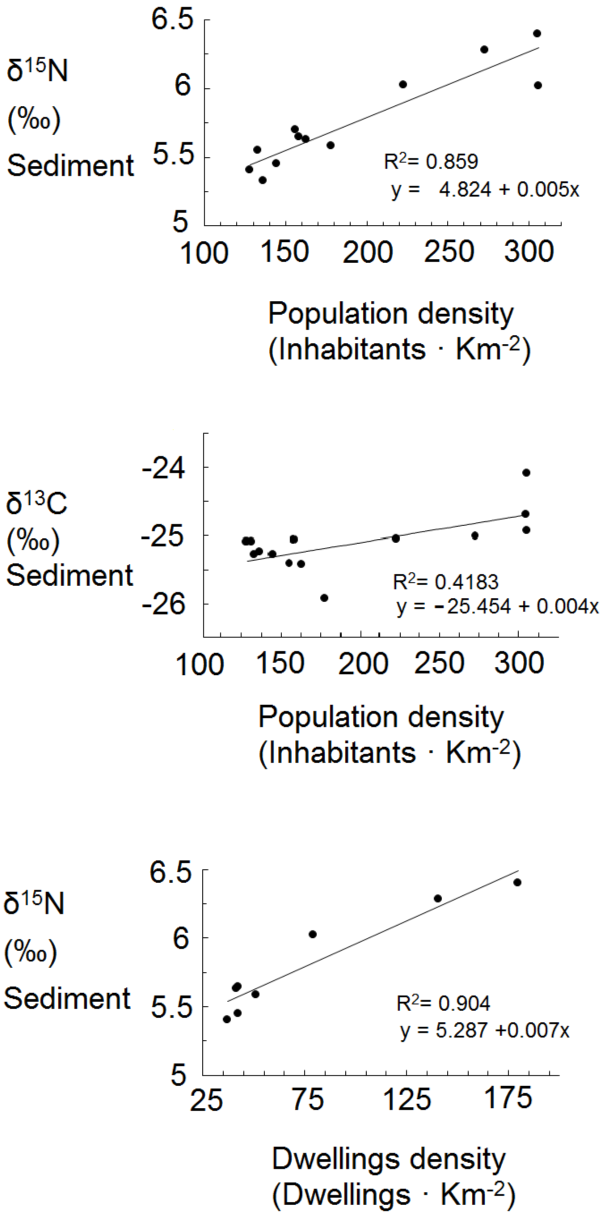

Fig. 8 Linear regression models built with $\delta^{15}N$ and $\delta^{13}C$ in core "A" from A Ramallosa vs. population and dwellings densities of the watershed corresponding to the ages of the isotopic ratios.