Non-indigenous species in soft-sediments: Are some estuaries more invaded than others?

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ABSTRACT

Non-indigenous species (NIS) are increasingly widespread and abundant in coastal areas, hence being considered indicators to assess the environmental status of marine waters under the Marine Strategy Framework Directive. However, information on the effects of biological invasions on species composition and abundance is still scarce, particularly for soft-sediment benthic communities, which remain poorly understood. Therefore, the present study aimed at monitoring the benthic communities of two Portuguese estuarine systems, with a particular focus on NIS.

Sampling surveys were conducted at the Tagus and Sado estuaries, in three different years. Invertebrate communities were collected at sampling stations located along the estuarine gradient, using a clam dredge, and several water and sediment parameters were measured at the same locations.

NIS represented nearly one fourth (23%) of the total observed individuals across all years and estuaries, with *Ruditapes philippinarum* accounting for 22% of the total abundance across estuaries and years. Although both estuaries presented a similar total number of species (91 at the Tagus and 81 at the Sado), three-fold more NIS were identified at the Tagus estuary. The frequency of occurrence of the NIS *Crassostrea (Magallana) gigas*, *Mya arenaria* and *Panopeus occidentalis* increased significantly over the three years at the Tagus estuary and decreased significantly for *Dyspanopeus sayi* from 2015 to 2018. The Tagus and Sado estuaries presented distinct and diverse communities, with the abundance of *R. philippinarum* being eleven-fold greater at the Tagus estuary than at the Sado estuary.

This study provides a baseline list of the NIS occurring in two of the main Portuguese estuarine systems, as well as an approach to estimate NIS abundance, richness, frequency of occurrence and community diversity. Although most studies on NIS impacts have focused on single species, the assessment of the environmental status of marine waters requires that overall impacts of NIS on marine ecosystems are determined. This study indicated metrics that are appropriate to evaluate changes in soft-sediment benthic communities associated to the introduction of NIS.

1. Introduction

Non-indigenous species (NIS) are increasingly widespread and abundant across all habitats, particularly in marine ecosystems (Bonanno and Orlando-Bonaca, 2019; Ruiz et al., 1999). Estuaries, bays and sheltered zones are especially vulnerable to biological invasions (Lejeusne et al., 2014; Ruiz et al., 1997; Young et al., 2017) when compared to exposed coastal areas, mainly due to the relatively high rate of human-mediated transfers (Ruiz et al., 1999, 2000, 2009).

Additionally, estuaries are focal points of human population, tourism, sea transport and trade, which may lead to unintentional introductions of alien species through the release of organisms transported in ballast water or fouling (Jimenez et al., 2018; Zorita et al., 2013). Although shipping (through ballast water and fouling) is considered the main introduction vector of marine NIS, other pathways include aquarium trade, aquaculture and fisheries releases (both intentional and unintentional) and the construction of navigation channels, floating debris or other structures (Ruiz et al., 2000).

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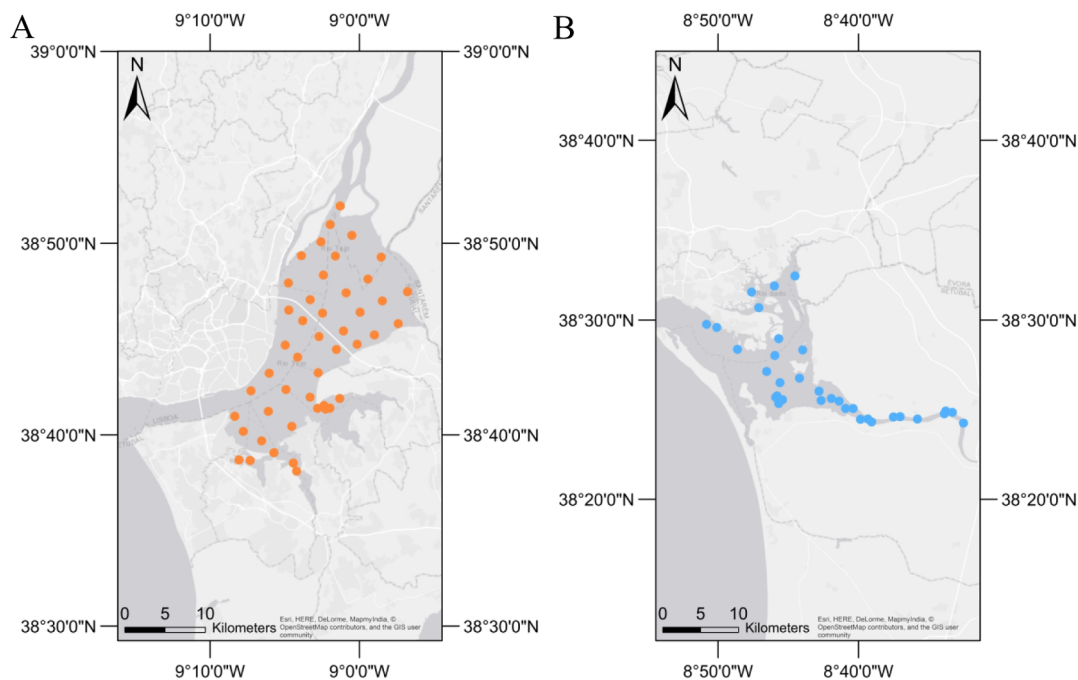
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Fig. 1. Location of sampling stations assessed in the A) Tagus Estuary and B) Sado Estuary. Orange and blue circles indicate the sampling stations at the Tagus and Sado estuaries, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The introduction of NIS is considered one of the major threats to the world's oceans, representing one of the main drivers of biodiversity loss (Jimenez et al., 2018; Ruiz et al., 1999; Streftaris et al., 2005; Zorita et al., 2013). The impacts are severe and interdisciplinary from ecological to economic (Kuprijanov et al., 2017; Zorita et al., 2013), including shifts in species' abundance, distribution and biotic interactions, changes on both habitat and community structures, modification of productivity and nutrient cycling, alteration of food chain resources and the introduction of pathogens (Jimenez et al., 2018; Lejeusne et al., 2010; Paavola et al., 2005; Streftaris et al., 2005; Young et al., 2017). In spite of being crucial to identify and assess these impacts, it is not always feasible to pinpoint and evaluate them directly. Biodiversity indices might be used as a proxy to the identification of overall impacts. Moreover, economic impacts are estimated at more than five per cent of the global economy (Burgiel and Muir, 2010; Lejeusne et al., 2014), with the cost for eradicating marine invasive species in Europe being at least 93.4 million € per year (Kettunen et al., 2008; Zorita et al., 2013). Consequently, species diversity, temporal occurrence, abundance and spatial distribution of NIS are indicators (descriptor D2 – non-indigenous species) used to assess the environmental status of marine waters under the Marine Strategy Framework Directive, which requires that "Non-indigenous species introduced by human activities are at levels that do not adversely alter the ecosystem" (MSFD, 2008/56/EC). Nevertheless, most of the baseline studies are conducted after biological invasions occur, with no reference conditions available to support the identification of adverse alterations in the ecosystem.

Despite the growing knowledge about NIS impacts, information on the cumulative effects of biological invasions on resulting community structure (particularly species composition and abundance) is still scarce (Jimenez and Ruiz, 2016). Moreover, the majority of studies concerning the occurrence of NIS in estuaries focus mainly on hard-substrates such as artificial structures at docks and marinas (Ruiz et al., 2009), whereas impacts on soft-sediment benthic communities remain poorly understood (Jimenez and Ruiz, 2016).

Among the Portuguese brackish water systems, the Tagus and Sado estuaries are the largest estuarine systems (França et al., 2009), as well as those with higher levels of anthropogenic pressure, such as population density, urbanization or global trade. Important shipping ports and

recreational marinas exist in both estuaries and an increase of activity in the aquaculture sector has been observed in the Sado estuary in the last ten years. Therefore, these two systems are highly susceptible to biological invasions, emphasizing the importance of studying and monitoring the biological communities with a particular focus on NIS.

The main objectives of this study were: i) to create the first comprehensive database of the existing NIS at both Tagus and Sado estuaries; ii) to quantify the overall contribution of NIS to soft-sediment benthic invertebrates species composition, abundance and community similarity; and iii) to identify temporal trends of biological invasions. It is expected that the outcomes of this study provide a science-based support for suitable management measures regarding NIS in these two estuarine systems.

2. Material and methods

2.1. NIS database for the Tagus and Sado estuaries

A thorough literature review was performed in order to identify and compile records of NIS at the Tagus and Sado estuaries with the aim of creating the first NIS database for these estuarine systems, including different habitats (i.e. soft-sediment communities, hard substrates, water column, etc.). Different sources of information were considered, such as scientific publications, technical reports, personal communications, unpublished reports, academic theses and the data collected during this study's sampling surveys (see below). All records were validated through the consultation of experts on the benthic communities of the Tagus and Sado estuaries and specialists on alien species. NIS have been categorized by Phyla and the nomenclature used is in accordance with the WoRMS (World Register of Marine Species, 2018) (www.marinespecies.org) and AlgaeBase (Guiry and Guiry, 2018). The year of first record and population status were indicated for each estuary, following criteria indicated by Chainho et al. (2015).

2.2. Study area

The Tagus and Sado estuaries were selected as study areas (Fig. 1). The first is the largest Portuguese estuarine system, with a total area of

320 km^2, a mean river flow of $300 \text{ m}^3.\text{s}^{-1}$ and a mean depth of 5 m. The second covers a total area of 180 km^2, presenting a mean river flow of $40 \text{ m}^3.\text{s}^{-1}$ and a mean depth of 6 m (França et al., 2009).

The Tagus estuary is a mesotidal estuary, with an average tidal range of 2.4 m, varying between 4 m and 1 m during spring and neap tides, respectively (Bettencourt et al., 2003). The salinity gradient along the estuary is strongly influenced by tidal and seasonal variations (Chainho, 2008) and water temperature ranges from 8 to 26 °C (Cabral et al., 2001). The intertidal flats and saltmarsh areas represent 40% of the total area of the Tagus estuary (Cabral et al., 2001) and are mainly composed of muddy areas and dead oyster beds (Granadeiro et al., 2007).

The Sado estuary is a mesotidal estuary (Bettencourt et al., 2003), with an average tidal range of 2.7 m (Rosa, 2010). The salinity gradient is highly influenced by tidal and seasonal cycles (Portela, 2016) and surface water temperature ranges from 10 to 26 °C (Cabeçadas et al., 1999). More than half of the area of this system is covered by wetlands, intertidal mudflats and saltmarshes (Garcês and Costa, 2009), presenting strong deflection of sandbanks dividing the lower estuary into two channels (Martins et al., 2001). Overall, most of this estuarine subtidal bottom is sandy (Rodrigues and Quintino, 1993).

2.3. Sampling methods

2.3.1. Sampling surveys

Sampling surveys were conducted in three different years at the Tagus estuary (May 2014, May 2015 and April 2018) and in two different years at the Sado estuary (July 2015 and May 2018). Sampling sites were randomly selected across the study area respectively at the Tagus (46 in total) and Sado (33 in total) estuaries, in order to be representative of different subtidal habitat types (Fig. 1). Samples were collected at each sampling site, in each estuary and year. Several physico-chemical parameters were measured at each sampling station, namely temperature (°C), depth (m), dissolved oxygen (%) and salinity, using an YSI multiparameter probe. Sediment samples were also collected using a van Veen grab (0.05 m^2) to determine the sediment grain size and the organic matter content (%). Furthermore, a clam dredge (Table 1) was towed once for 30 s at a mean speed of 1.5 knots in every sampling station, on board of a professional fishing vessel, to collect invertebrate organisms larger than 2 cm. Collected specimens were sorted onboard and placed in labelled plastic bags.

2.3.2. Laboratory procedures

Biotic samples were processed in the laboratory, where the specimens were identified to the species level, counted, measured and weighted. Several books and scientific papers on taxonomy were used to perform the identifications, since the benthic Portuguese fauna

Table 1
Major characteristics of the clam dredge used to collect invertebrate organisms larger than 2 cm.

Gear specifications	Dimensions (cm)
Shaft	
Diameter	80.0
Width	60.0
Height	30.0
Tooth bar	
Number of teeth	13.0
Tooth spacing	1.5
Tooth thickness	1.0
Tooth length	12.0
Net bag	
Length	230.0
Width	70.0
Mesh size	3.0

includes Atlantic and Mediterranean species. Moreover, the identification of NIS required the use of additional literature specific for NIS. Sediment samples were dried at 60 °C for 24 h to determine the total organic matter (%) and sediment grain size. Total organic matter was determined as the difference between dry weight (measured after drying the sample at 60 °C during 24 h) and ash weight (obtained after ignition in a muffle furnace at 550 °C for a period of 4 h). The sediment composition was estimated by calculating grain size parameters (mean phi value for each sample) according to Folk & Ward (1957) formulae using GRADISTAT v8 and taking into consideration the weight retained in four "AFNOR" sieves with the following apertures: 2 mm, 0.5 mm, 0.25 mm, 0.063 mm.

2.4. Data analysis

The NIS richness (%) and the mean percentage of occurrence of NIS in each estuary were estimated and differences among years were tested (Table 2). The relative abundance and contribution of NIS to both number and percentage of species recorded in each sampling station of each estuary and in each year were compared using the non-parametric Friedman test (significance level $p < 0.05$) and the *a posteriori* Bonferroni test performed on IBM SPSS Statistics v.25. The frequency of occurrence of each NIS was also estimated as the number of sampling stations in which it was recorded across all sampling stations, for each year and in each estuary and a Pearson's chi-square test for independence (significance level $p < 0.05$) was performed to test if there were significant differences between years (Table 2).

Bray-Curtis similarity matrices were calculated for the benthic community composition based on square root transformed abundance data for all species. Spatial and temporal patterns of the benthic communities were analyzed through a Principal Coordinate Ordination (PCO) conducted on data collected in 2015 and 2018 at the Tagus and Sado estuaries. To understand patterns of association, vectors of species (Pearson correlation coefficient r > 0.4) and environmental data (all variables included) were overlaid in the PCO graph.

Differences in the benthic community's composition among years and estuaries were tested using a Permutational Analysis of Variance (PERMANOVA) with a two-way factorial design: Year (fixed, 2 levels: 2015 and 2018) nested in Estuary (fixed, 2 levels: Tagus estuary and Sado estuary), using only the years in common between the two estuaries. A similarity percentage analysis (SIMPER) was performed to identify the species that contributed mostly for similarities within estuaries and dissimilarities between estuaries and between years. PCO, PERMANOVA and SIMPER routines were conducted using PRIMER v.6 with Permanova + add on (Anderson et al., 2008; Clarke and Gorley, 2006).

3. Results

3.1. NIS database

A total of 60 NIS included in 11 different Phyla was recorded in all habitats at the Tagus and Sado estuaries (Table 3), with a higher number of NIS registered at the Tagus estuary (56), when compared to the Sado estuary (27). Twenty-five of these NIS are soft-sediment species or might be associated with soft-sediment species as fouling, representing nearly half of the total NIS listed. The oyster *Crassostrea (Magallana) gigas* was the first NIS to be registered in both estuaries (< 1700 and 1960 at the Tagus and Sado estuaries, respectively) and two NIS were new records in 2018 (*Ocinebrellus inornatus* and *Panopeus occidentalis*).

3.2. Abundance

NIS collected during sampling surveys accounted for 23% of the 59,813 specimens collected across estuaries and years. At the Tagus and

Table 2

List of metrics estimated for NIS. For each metric a clear definition, the statistical test used to determine significant temporal variations and the associated MSFD indicator is specified. The significance level considered was $p < 0.05$ for all the statistical tests.

Metric	Definition	Statistical test	MSFD Indicator
NIS richness/station	For each station: Number of NIS	Friedman test	Species diversity
NIS richness/station (%)	For each station: (Number of NIS/Total number of species) × 100	Friedman test	Species diversity
Relative NIS abundance/station (%)	For each station: (Number of individuals of NIS/Total number of individuals) × 100	Friedman test	Abundance
Mean contribution of NIS (%)	Average of all percentages of NIS richness/station	–	Species diversity
NIS richness (%)	(Number of NIS/Total number of species) × 100	Chi-square test	Species diversity
Frequency of occurrence/NIS (%)	For each NIS: Number of stations where occurred/Total number of stations	Chi-square test	Spatial distribution
Mean percentage of occurrence	Average of all frequencies of occurrence of each NIS	Chi-square test	Spatial distribution

Sado estuaries, NIS represented 28% and 4% of the community abundance, respectively. The Manila clam *Ruditapes philippinarum* was the most abundant NIS across estuaries and years, representing nearly 22% of total abundance. The NIS relative abundance per station was not significantly different over the years at either the Tagus or the Sado estuaries (Friedman test $p = 0.282$ and $p = 0.549$, respectively). The relative sampling effort at the Tagus estuary was 162 individuals/30 s, 264 individuals/30 s and 565 individuals/30 s in 2014, 2015 and 2018, respectively; whereas at the Sado estuary the relative sampling effort was 209 individuals/30 s and 220 individuals/30 s in 2015 and 2018, respectively.

3.3. Species richness

A total of 127 species (116 native and 11 NIS) was identified across all sampling stations, years and estuaries, including 91 species at the Tagus estuary and 81 species at the Sado estuary. Three NIS were detected at the Sado estuary (*Chaetopleura angulata*, *Dyspanopeus texanus* and *Ruditapes philippinarum*), whereas at the Tagus estuary ten NIS were identified (*Arcuatula senhousia*, *C. angulata*, *D. sayi*, *Eriocheir sinensis*, *Crassostrea (Magallana) gigas*, *Mya arenaria*, *Ocinebrellus inornatus*, *Panopeus occidentalis*, *Rhithropanopeus harrisii* and *Ruditapes philippinarum*). Only 43 out of the 91 species (34%) occurred in both estuaries and only two of these were NIS (*C. angulata* and *R. philippinarum*). In each year, the NIS occurred in at least 32 and 15 of the 46 and 33 stations at the Tagus and Sado estuaries, respectively.

The NIS richness (%) showed a trend to increase over the three years at the Tagus estuary (Fig. 2), although no significant differences were found ($p = 0.187$) reaching the highest value in 2018 (nearly 13%). Contrarily, at the Sado estuary the NIS richness (%) showed a trend to decrease between 2015 and 2018, but once more no significant differences were found ($p = 0.226$).

The mean percentage of occurrence of NIS showed a similar pattern as the NIS richness (%): in spite of a trend to increase along the three years at the Tagus estuary and decrease between 2015 and 2018 at the Sado estuary, these variations were not statistically significant ($\chi^2 = 1.180$, $p = 0.554$ and $\chi^2 = 0.449$, $p = 0.503$ for the Tagus and Sado estuaries, respectively). Nevertheless, it is important to note that the mean percentage of occurrence of NIS at the Sado estuary was always higher when compared to the Tagus estuary, in the same year (Fig. 3).

At the station level, the mean contribution of NIS (%) to the species richness across all years was approximately 27% at the Tagus estuary (ranging from 0 to 100%), whereas at the Sado estuary this value was close to 10% (ranging from 0 to 50%). Moreover, the NIS richness/station at the Tagus was significantly different between years ($p = 0.001$), with significant differences between 2014 and 2018 (Friedman Bonferroni adjusted $p = 0.022$). No statistically differences in NIS richness/station were found between 2015 and 2018 at the Sado estuary ($p = 0.317$). No significant differences were found between years for the NIS richness/station (%), at both the Tagus and the Sado estuaries ($p = 0.472$ and $p = 0.102$, respectively).

The frequency of occurrence of some species present at the Tagus estuary increased significantly over the three years: *C. (Magallana) gigas* ($p = 0.013$), *M. arenaria* ($p = 0.022$) and *P. occidentalis* ($p < 0.001$) and decreased significantly from 2015 to 2018 for *D. sayi* ($p < 0.001$). On the other hand, the frequency of occurrence was not significantly different among years for any of the NIS observed at the Sado estuary.

3.4. Community diversity

The first two axes of the PCO (Fig. 4) concerning the soft-sediment macrofauna community present at the Tagus and Sado estuaries in the different years accounted for 15.4% and 12.7% of the variation in the dataset, respectively. Although the ordination does not show a clear segregation between estuaries or years, most stations from the Tagus estuary are represented on the negative axis of PCO1, with a strong association to higher abundances of *Ruditapes philippinarum* but no clear associations with environmental parameters. On the other hand, stations from the Sado estuary are predominantly positioned on the positive axis of PCO1. Additionally, there seems to be a separation between upstream and downstream sampling stations along the second axis, with the uppermost sites positioned in the negative axis and associated to a higher abundance of *Scrobicularia plana* and environmental conditions characterized by muddy sediments (higher phi values) with higher organic content (TOM). Otherwise, downstream sampling stations are mostly positioned on the positive axis of PCO2, associated to higher abundances of *Ostrea stentina* and higher depths and salinities (Fig. 4).

The PERMANOVA analysis showed that significant differences between estuaries and years were found (Table 4). Additionally, the SIMPER analysis indicated high average dissimilarity percentages between the Tagus and the Sado estuarine invertebrate communities in 2015 and 2018 (Fig. 5) with the NIS *R. philippinarum* as the species that contributed most for this dissimilarity (17.37% and 16.08% in 2015 and 2018, respectively). Furthermore, the average abundance of *R. philippinarum* was always higher in the Tagus estuary (6.01 in 2015 and 8.02 in 2018) than in the Sado estuary (1.70 in 2015 and 1.40 in 2018), regardless of the year.

The similarity of species composition and abundance within each estuary in each year was relatively low (< 22%), but the overall benthic composition structure was more homogenous at the Tagus estuary than at the Sado estuary. The NIS *R. philippinarum* contributed most for these similarities except for the Sado estuary in 2018, when the native species *O. stentina* and *Solen marginatus* had a major contribution. Moreover, each estuary presented high dissimilarity percentages between years (> 80%) with *R. philippinarum* and *O. stentina* being the most relevant species for the Tagus and Sado estuaries, respectively. The average abundance of *R. philippinarum* in the Tagus estuary increased between years (6.01 in 2015 and 8.02 in 2018), similarly to the average abundance of *O. stentina* in the Sado estuary (3.70 in 2015 and 5.56 in 2018).

4. Discussion

Non-indigenous species are increasingly conspicuous worldwide,

Table 3

List of NIS recorded at the Tagus and Sado estuaries in all habitats. The year of first record (underscored for species which have been observed during this study) and populations status (E-Established, NE-Not Established, U-Undetermined) are indicated for each estuary, as well as references used as information sources (in the last two cases separated by semicolons for the Tagus and Sado estuaries). *Soft-sediment species.

Taxa	Year of first record		Population status	References
	Tagus	Sado		
Chlorophyta				
Codium fragile (Suringar) Hariot, 1889	2010		NE	Chainho et al. (2015)
Ochrophyta				
Colpomenia peregrina Sauvageau, 1927	2003		E	Berecibar (2011)
Undaria pinnatifida (Harvey) Suringar, 1873	2016	2017	U; U	Berecibar (2016) and P. Chainho (pers. com.)
Rhodophyta				
Anotrichium furcellatum (J. Agardh) Baldock, 1976	2016	2016	E; E	Berecibar (2011) and Berecibar (2016)
Antithamnion amphigeneum A. Millar, 1990	2008	2016	E; E	Berecibar (2011) and Berecibar (2016)
Antithamnion densum (Suhr) M.A. Howe, 1914	2016		E	Berecibar (2016)
Antithamnion hubbsii E.Y. Dawson, 1962	2016		E	Berecibar (2016)
Antithamnion nipponicum Yamada & Inagaki, 1935	2010		NE	Berecibar (2011)
Antithamnionella spirographidis (Schiffner) E.M. Wollaston, 1968	2016		E	Berecibar (2016)
Antithamnionella ternifolia (J.D. Hooker & Harvey) Lyle, 1922	1963		E	Ardré (1969)
Asparagopsis armata Harvey, 1855	1957		E	Ardré (1969)
Dasya sessilis Yamada, 1928	1989		E	Berecibar (2011)
Gracilaria vermiculophylla (Ohmi) Papenfuss, 1967	1989		E	Berecibar (2011)
Grateloupia filicina (J.V. Lamouroux) C.A. Gardh, 1822	1963		E	Ardré (1969)
Lomentaria hakodatensis Yendo, 1920	2016		U	Berecibar (2016)
Melanothamnus harveyi (Bailey) Díaz-Tapia & Maggs, 2017	2003		E	Berecibar (2011)
Symphyocladia marchantioides (Harvey) Falkenberg, 1897	2003		E	Berecibar (2011)
Tracheophyta				
Spartina patens (Aiton) Muhl*	1999	1999	E; E	SanLeón et al. (1999) and SanLeón et al. (1999)
Cnidaria				
Cordylophora caspia (Pallas, 1771)	2011		U	Conde et al. (2013a)
Ctenophora				
Mnemiopsis leidyi A. Agassiz, 1865		2017	U	ICES (2018)
Annelida				
Desdemona ornata Banse, 1957*	2009		E	Costa et al. (2010)
Ficopomatus enigmaticus (Fauvel, 1923)*	1987		E	M. Guerra & M. Gaudêncio (pers. com.)
Prionospio pulchra Imajima, 1990*	2012		E	G. Silva (pers. com.)
Pseudopolydora paucibranchiata (Okuda, 1937)*	2005		E	G. Silva (pers. com.)
Bryozoa				
Bugula neritina (Linnaeus, 1758)	2017	2013	E; E	Afonso (2018) and Unpublished data
Tricellaria inopinata d'Hondt & Occhipinti Ambrogi, 1985	2017		E	Afonso (2018)
Watersipora subtorquata (d'Orbigny, 1852)	2009	2013	E; E	Chainho et al. (2015) and Unpublished data
Mollusca				
Arcuatula senhousia (Benson, 1842)*	2018	2015	U; U	This work; ICES (2018)
Chaetopleura angulata (Spengler, 1797)*	1987	1979	E; U	Gaudêncio and Cabral (2007) and Cancela da Fonseca et al. (1987)
Corbicula fluminea (O. F. Müller, 1774)*	1978	2015	E, E	C. Reis (pers. com.) and P. Chainho (pers. com.)
Crassostrea (Magallana) gigas (Thunberg, 1793)*	1700	1960	E; E	Edwards (1977) and Ruano and Sobral (2000)
Mercenaria mercenaria (Linnaeus, 1758)*		2010	NE	Miguel Gaspar (pers. com.)
Mya arenaria Linnaeus, 1758*	2007	1983	E; U	Conde et al. (2010) and Gamito (1983)
Ocinebrellus inornatus (Récluz, 1851)*	2018		U	This work
Potamopyrgus antipodarum (Gray, 1843)*	1988		E	Costa et al. (1992)
Ruditapes philippinarum (Adams & Reeve, 1850)*	2000	2010	E; E	Gaspar (2010) and Gaspar (2010)
Tonicia atrata (G.B. Sowerby II, 1840)*		1985	U	Arias and Anadón (2013)
Arthropoda				
Acartia (Acanthacartia) tonsa Dana, 1849	1985	1995	E; U	Sobral (1985) and Monteiro et al. (1995)
Amphibalanus amphitrite (Darwin, 1854)*	2017		E	Afonso et al. (2019)
Amphibalanus improvisus (Darwin, 1854)*	1982		E	Calvário (1982)
Austrominius modestus (Darwin, 1854)*	2010	1998	E; NE	Afonso et al. (2019) and O'Riordan and Ramsay (1999)
Balanus trigonus Darwin, 1854*	2017	2013	E; E	Afonso et al. (2018) and Chainho et al. (2015)
Callinectes sapidus Rathbun, 1896*	1978	1993	NE; U	Gaudêncio and Guerra (1979) and Ribeiro and Veríssimo (2014)
Caprella scaura Templeton, 1836	2016	2011	E; U	Afonso et al. (2019) and Cabezas et al. (2014)
Dyspanopeus sayi (Smith, 1869)*	2015		U	This work
Dyspanopeus texanus (Stimpson, 1859)*		2015	U	This work
Eriocheir sinensis H. Milne Edwards, 1853*	1992		E	Cabral and Costa (1999)
Megabalanus tintinnabulum (Linnaeus, 1758)	2016	2016	U; U	Guerra and Gaudêncio (2016) and Guerra and Gaudêncio (2016)
Palaemon macrodactylus Rathbun, 1902	2017		NE	Afonso (2018)
Panopeus occidentalis Saussure, 1857*	2018		U	This work
Penaeus japonicus Spence Bate, 1888	2017	1985	U; U	A. Santos (pers. com.) and Ruano and Sobral (2000)
Rhithropanopeus harrisii (Gould, 1841)*	1978		NE	Projecto-Garcia et al. (2010)
Chordata				
Botrylloides violaceus Oka, 1927	2016		U	Ramos-Esplá (2016)
Botryllus schlosseri (Pallas, 1766)	2016		U	Ramos-Esplá (2016)
Corella eumyota Traustedt, 1882	2009		E	El Nagar et al. (2010)

Table 3 (*continued*)

Taxa	Year of first record		Population status	References
	Tagus	Sado		
Cynoscion regalis (Bloch & Schneider, 1801)	2017	2015	U; U	Morais et al. (2017) and Béarez et al. (2016)
Microcosmus squamiger Michaelsen, 1927	2017	2018	E, U	Afonso (2018); P. Chainho (pers. com.)
Molgula manhattensis (De Kay, 1843)	2016	2018	E, E	Ramos-Esplá (2016); P. Chainho (pers. com.)
Styela cf. *plicata* (Lesueur, 1823)	2016		E	Afonso (2018)
Styela clava Herdman, 1881	2003	2016	E; U	Davis and Davis (2005) and Unpublished data
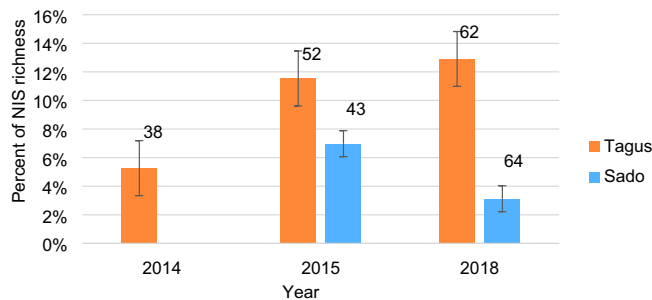
Fig. 2. NIS richness percentage $\left(\frac{Number \ of \ NIS}{Total \ number \ of \ species} \times 100 \right)$ in each year and estuary, collected with a clam dredge. Bars represent the standard error. Numbers on top of bars indicate the total species richness per year and estuary.
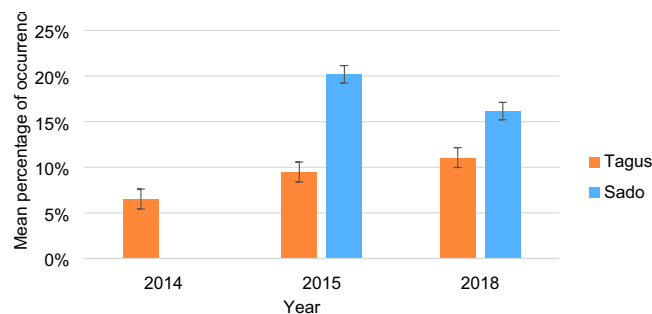

Fig. 3. Mean percentage of occurrence of NIS (average of all frequencies of occurrence of each NIS) in each year and estuary, collected with a clam dredge. Bars represent the standard error.

Fig. 4. Principal Coordinate Ordination (PCO) of benthic communities' abundance at the Tagus and Sado estuaries based on Bray-Curtis similarities. Lengths of overlaid vectors indicate the Pearson correlations coefficient of each species ($r > 0.4$) and environmental variable (all variables included) with the two first PCO axes.

Table 4
PERMANOVA analysis results on transformed (square-root) abundances of macrofauna (Bray-Curtis similarity). Estuary is a fixed factor (2 levels: Tagus and Sado) and Year is a fixed factor (2 levels: 2015 and 2018) nested in the Estuary (df: degrees of freedom; MS: mean square).

Source	df	MS	Pseudo-F	P (perm)
Estuary	1	31,862	8.7359	0.001
Year (Estuary)	2	8513.7	2.3343	0.001
Residuals	147	3647.2		

particularly in Europe where the highest number was reported (Katsanevakis et al., 2014). However, some European countries, such as Portugal, have a low number of NIS recorded and do not have a national online database on alien species, mainly due to lack of monitoring and reporting efforts (Katsanevakis et al., 2013). In addition, although there are some studies focusing on marine NIS in Portugal (Afonso, 2011; Cabral and Costa, 1999; Conde et al., 2010), these usually address single species, while information regarding marine NIS communities remains scarce. Therefore, this study presents the first comprehensive database on NIS occurrence at the Tagus and Sado estuaries. These two systems recorded nearly half of the 166 species catalogued for the Portuguese coastal areas, estuaries, coastal lagoons and islands (Chainho et al., 2018), indicating that they are key systems to monitor and study concerning biological invasions. Furthermore, a considerable effort has been focused on hard-substrates worldwide, while relatively little information is available on soft-sediment benthic macrofauna (Jimenez and Ruiz, 2016). Nevertheless, the database compiled in this study showed that soft-sediment species accounted for nearly half of the reported NIS in these estuaries, reinforcing the importance of assessing soft-sediment habitats in order to reduce the gap of knowledge on biological invasions. The oldest NIS record in both estuaries was *Crassostrea (Magallana) gigas*, which introduction is associated with the Portuguese old shipping routes and colonial history
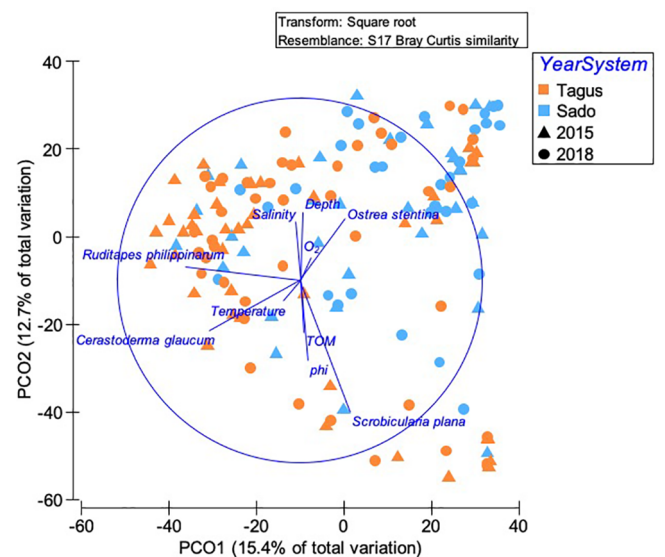
(Chainho et al., 2015) and the import of this species from Japan in the 16th century (Edwards, 1977).

In the current survey, NIS represented nearly one fourth of the total specimens identified across all years and estuaries, which can be considered as a relatively low level of invasion when compared to other areas of the world, such as San Francisco Bay, where 76% of all detected organisms were NIS (Jimenez and Ruiz, 2016). However, it is important to note that San Francisco Bay is one of the most highly invaded bays in the world (Jimenez and Ruiz, 2016), as well as one of the best studied coastal bays in what regards research and assessment of marine NIS over many decades (Alpine and Cloern, 1992; Cloern et al., 2011; Grosholz et al., 2000; Lee et al., 2003), whereas the Tagus and Sado estuaries were monitored only in the last decade. The Manila clam *Ruditapes philippinarum* accounted for approximately a quarter of the total abundance, across estuaries and years, being currently the dominant bivalve species at the Tagus estuary and one of the most abundant bivalves at the Sado estuary (Chainho et al., 2015; Chiesa et al., 2017;
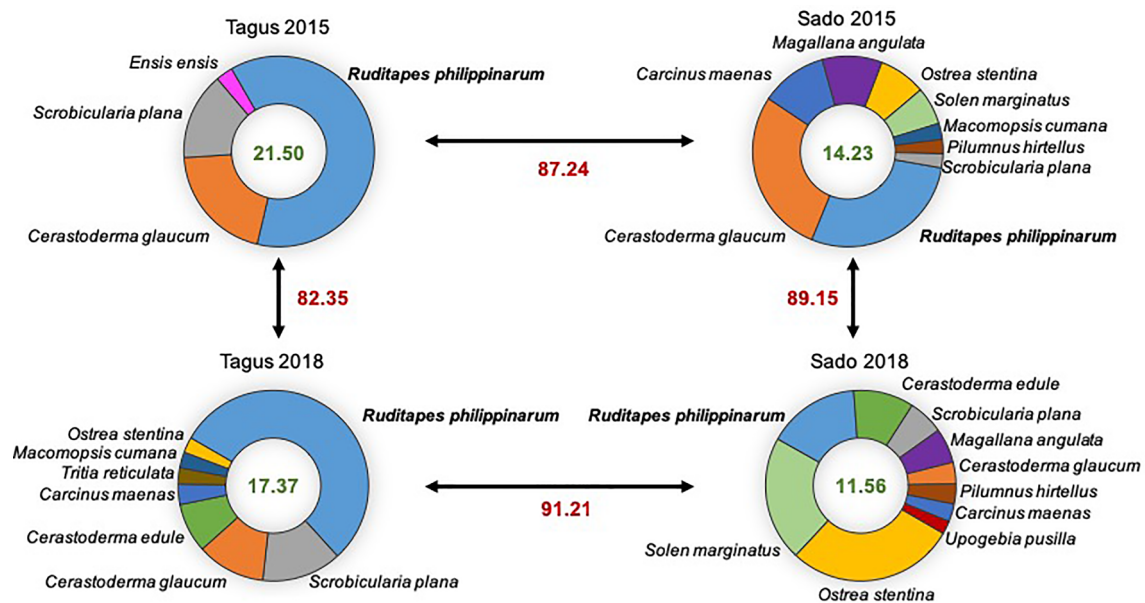
Fig. 5. Graphical representation of the SIMPER analysis for the benthic communities' abundance in the Tagus and Sado estuaries for 2015 and 2018. Each graph represents the relative contribution of each species to similarities within each estuary and year. The overall similarity percentage within samples of the same estuary and year is indicated inside each graph in green, whereas the dissimilarity percentage between estuaries (for the same year) and between years (for the same estuary) is indicated in red by the arrows. Species in bold are NIS. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

ICES, 2014).

Concerning species richness, both estuaries showed a similar total number of species, but the Tagus estuary recorded three-fold more NIS than the Sado estuary. One factor that might explain this difference is higher introduction pressure (i.e. introduction pathways) present at the Tagus estuary when compared to the Sado estuary. Shipping is considered the major vector of introduction of marine NIS in Portugal (Chainho et al., 2015) and 15 shipping ports (including cruise terminals, container ports, bulk ports and multipurpose terminals, among others) and 8 recreational docks (with a total berth capacity for 2502 vessels) (Afonso, 2018; Porto de Lisboa, 2019) are located in the geographical range area of the Tagus estuary. On the other hand, 12 shipping ports (including container ports, bulk ports and multipurpose terminals, among others) and 7 recreational docks (with a total berth capacity for 814 vessels) (Porto de Setúbal, 2019) are located in the geographical range area of the Sado estuary. Although the number of infrastructures is not so different, the number of vessels varies considerably and the cargo volume for these two ports was also very distinctive during the years of this study: 10,766,164 tons (2014), 10,527,867 tons (2015) and 11,149,425 (2017) for the Port of Lisbon (Tagus estuary); and 7,808,194 tons (2014), 7,221,709 tons (2015) and 5,814,763 tons (2017) for the Port of Setúbal (Sado estuary) (AMT, 2018). Hence, these numbers suggest that the Tagus estuary is more susceptible to host NIS that may arrive through ballast water of these cargo ships or through fouling on commercial and recreational vessels.

NIS richness per station was significantly higher in 2018 at the Tagus estuary, suggesting that the number of NIS increased over the three years at different locations within this system. Nevertheless, the representativeness of NIS per station was not significantly different over the years, indicating that not only the number of NIS, but also the number of native species increased in these years. These differences might be associated to inter annual variations in the environmental and biological conditions. It might also be related to the selectivity of the sampling device used, which targeted mainly bivalve and sedentary species, but several mobile species were also recorded, which may represent circumstantial occurrences.

The bivalves *C. (Magallana) gigas* and *Mya arenaria* and the crab *Panopeus occidentalis* significantly increased their frequency of occurrence over the three years at the Tagus estuary, indicating a possible extension of their spatial distribution area. The first record of *C. (Magallana) gigas* in the Tagus estuary dates back to the 16th century (Carlton, 1999; Edwards, 1977). Therefore, this study indicates that *C. (Magallana) gigas* might be expanding its population at the Tagus estuary, after the mass mortalities and disappearance in the 1970's (Batista, 2007). *Mya arenaria* was registered in the Tagus estuary in 2007 (Conde et al., 2010) and an increase in the number of stations where it occurred was observed over the years, indicating an expansion of its spatial distribution, as it was previously referred (Conde et al., 2013b; Garaulet, 2011). Finally, *P. occidentalis* was detected for the first time at the Tagus estuary only in 2018, occurring in more than 13% of the sampling sites, suggesting that it was introduced in the later years, but its distribution area is expanding very fast in this system. Some NIS may become invasive species since they have several attributes, such as wide physiological tolerances, high genetic variability, short generation time, early sexual maturity, high reproductive capacity and a broad diet (e.g. Essink and Dekker, 2002; Lejeusne et al., 2014). Different scenarios can be observed after the introduction of a NIS: 1) the species may remain rare and with low abundances; 2) it may become invasive and, in the subsequent years, may have a reduction in spatial distribution and abundance; or 3) after becoming invasive, the species may be dominant in the ecosystem, hampering the endeavor of eradication. Thus, *P. occidentalis* may have a wide distribution range in the subsequent years of the introduction. Otherwise, the frequency of occurrence of *Dyspanopeus sayi* (which was registered at the Tagus estuary in 2015) significantly decreased from 2015 to 2018. These few records along with the fact that this species was not observed in 2018 may indicate that either the population of this NIS is not established in this system, or it has an established population but with low abundances, as referred for other species by some authors (Chainho et al., 2015; Ribeiro and Veríssimo, 2014).

In what concerns the sampled soft-sediment community, the Tagus and Sado estuaries showed distinct and diverse communities. These differences might be related not only to the area of the estuarine systems, with the Tagus occupying a significantly higher area, but also to the invasion status of each estuary, with a much higher number of NIS occurring at the Tagus estuary. *Ruditapes philippinarum*,

Scrobicularia plana, Ostrea stentina and *Cerastoderma glaucum* were identified as the most relevant species in the community structure in these estuarine systems, emphasizing the fact that an invasive species can become a key species in a short time after its introduction (≈18 years). *Ruditapes philippinarum* occurred in more than half of the locations sampled at the Tagus estuary, while at the Sado estuary it was only recorded in one fourth of the sampling stations. Furthermore, the abundance of *R. philippinarum* was eleven-fold higher in the Tagus than in the Sado estuary, indicating that this NIS is a crucial component of the Tagus macrofauna community. These differences might be associated to a different invasion stage in each of these two brackish water systems (Chan et al., 2012; Colautti and MacIsaac, 2004). In fact, according to Gaspar (2010), *R. philippinarum* was introduced at the Tagus estuary 10 years earlier than at the Sado estuary, where it was firstly detected in 2010. Consequently, at the time this study was conducted (2014, 2015 and 2018) the population was likely established at the Tagus estuary but still in an expansion stage at the Sado estuary. Furthermore, after an adaptation period that can vary between three and five years (Gaspar, 2010), this species generally undergoes a demographic explosion in population (Pranovi et al., 2006), hence justifying the increase in abundance that occurred at the Tagus estuary throughout the years, whereas not yet changing considerably at the Sado estuary. In conclusion, these results indicate that the Tagus estuary is more invaded than the Sado estuary, both in terms of number and abundance of NIS. Nevertheless, since both systems have a diverse habitat availability and high levels of pressure related to major introduction vectors (e.g. shipping and aquaculture), an increase on the invasion status might occur in the Sado estuary. A continued monitoring of these systems is essential to understand the invasion process and estimate other potential areas of invasions within systems where *R. philippinarum* occurs and others that might be invaded in future, namely using predicting models based on environmental variables.

The interpretation of the results of this study is conditioned by the constraints of the sampling device used to collect samples, since it targets mainly benthic sedentary organisms larger than 2 cm. Future studies comparing results obtained using clam dredges and grabs would be helpful to understand possible limitations associated to the sampling device.

This study provides a baseline list of the NIS present in two of the main Portuguese estuarine systems, as well as an approach to estimate NIS abundance, richness, frequency of occurrence and community diversity. These metrics were used in order to create a time-series database and evaluate temporal trends in NIS. Despite being included in the Marine Strategy Framework Directive (MSFD) as a specific qualitative descriptor, the temporal occurrence, abundance and spatial distribution of NIS in a marine region may not be the most appropriate indicators alone to determine the good environmental status of marine waters because evaluating the impacts and metrics of a single NIS alone is not equivalent to assessing the overall community (including multiple NIS and/or native versus NIS combined). Consequently, other metrics should also be determined such as the contribution of NIS to the community structure. The MSFD provides only broad guidelines regarding this descriptor and therefore, it is crucial to implement specific and standardized approaches in order to ensure the efficiency and the accuracy of the assessment. Likewise, the continuous monitoring of these metrics becomes of the utmost importance.

Several management and legislative measures were adopted by the European Union to address NIS, though most of them are yet voluntary codes of conduct. One of these strategies is the BWM Convention (International Convention on the Control and Management of Ships' Ballast Water and Sediments) that aims to prevent potential negative effects resulting from the introduction of NIS carried by ships' ballast water on different regions. However, this convention did not entry into force in all countries yet, hampering its effectiveness in reducing the spread of NIS worldwide. Other legislative actions include a regulation focusing on the use of non-indigenous and locally absent species in aquaculture ((EC) No 708/2007 of 11 June 2007) and a list of invasive alien species of Union concern that are subject to restrictions and measures clearly defined ((Regulation EU) 1143/2014). In spite of being crucial steps to mitigate NIS introductions, these policies are only effective when implemented and complied, so surveillance should be enforced.

It is highly recommended that future studies further investigate NIS community across different types of habitats (rocky-bottoms, artificial structures, soft-sediment), since it is essential to not under or over-estimate the proportion of NIS in estuarine systems (Jimenez et al., 2018) and because previous studies indicate that NIS colonization is greater on hard-bottoms rather than soft-bottoms (Ruiz et al., 2009). Moreover, effective management measures to mitigate NIS impacts and reduce introductions must therefore be implemented.

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