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Effects of the non-indigenous bivalve *Ruditapes philippinarum* on meiofaunal communities of the Tagus estuary

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Ignorance more frequently begets confidence than does knowledge: it is those who know little, and not those who know much, who so positively assert that this or that problem will never be solved by science.

Charles Darwin

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Contents

Efeitos do bivalve não-indígena *Ruditapes philippinarum* nas comunidades de meiofauna do estuário do Tejo.

|RESUMO

A meiofauna é, atualmente, considerada como um grupo de organismos com elevado potencial para a monitorização de eventuais efeitos ecológicos resultantes de perturbações naturais e/ou antropogénicas nos ecossistemas aquáticos. A presença do bivalve não-indígena *R. philippinarum* no estuário do Tejo apresentou uma oportunidade para avaliar o uso das comunidades da meiofauna, particularmente os nematodes, como bioindicadores que permitam avaliar perturbações provocadas pela presença de uma espécie alóctone.

O principal objetivo desta investigação foi verificar as respostas estruturais e funcionais das comunidades de meiofauna perante a presença de um bivalve não-indígena. Este objetivo foi atingido através da comparação de padrões de densidade, diversidade e composição trófica das comunidades de meiofauna, especialmente os nematodes, em locais com diferentes abundâncias de *R. philippinarum*. Todas as análises realizadas mostraram que a densidade, os padrões de distribuição, diversidade e a composição trófica das comunidades de nematodes foram estruturados principalmente por fatores ambientais como o tipo de sedimento e a salinidade e não tanto pelas densidades de *R. philippinarum*.

Effects of the non-indigenous bivalve *Ruditapes philippinarum* on meiofaunal communities of the Tagus estuary.

|ABSTRACT

Meiofauna are presently regarded as very suitable organisms to monitor potential ecological effects of natural and anthropogenic disturbances in aquatic ecosystems. The presence of the non-indigenous bivalve *R. philippinarum* in the Tagus estuary presented the opportunity to evaluate the ability of meiofauna communities, specifically nematodes, as indicators to assess disturbances promoted by an allochthonous species.

This research focused on the benthic meiofauna assemblages response to the presence of a non-indigenous bivalve. Density, diversity and trophic composition patterns of the meiofauna communities, particularly nematodes were examined along the estuary gradient and related to environmental conditions and the occurrence of *R. philippinarum*. All the performed analysis showed that nematodes density, distribution patterns, diversity and trophic composition were mainly structured by distinct environmental factors like sediment grain size and salinity rather than *by R. philippinarum* densities.

Meiofauna: definition, distribution, abundance, biological characteristics and ecological role

The term meiofauna (commonly used as a synonym for meiobenthos) is derived from the Greek word “μειος” meaning “smaller”, and encompasses the metazoan (some investigators also include the foraminiferans in the meiofauna definition) component of the benthos smaller than the macrofauna (Coul, 1999; Giere, 2009). The term “meiobenthos” was first proposed by Mare (1942) as a necessity to use a new terminology equivalent to the macrobenthos under which small organisms such as nematodes, copepods and others that occurred on the bottom but that were not regarded as true microbenthos could be included. The term meiofauna loosely defines a group of organisms by their size rather than a taxonomic grouping. Even though the variation in form and the biological complexity of the meiofauna makes it difficult to determine upper and lower limits in terms of body size which can be applied to all taxa, it is very helpful for the standardization methods of work for the taxonomists and ecologists studying these organisms (Hulings & Gray, 1971).

The formal size boundaries of meiofauna are operationally defined based on the standardized mesh width of sieves with 1000 µm (or 500 µm) as upper and 63 µm (or 44 µm) as lower limits: all fauna that pass through the coarser sieve but are retained by the finer sieve are considered meiofauna. More recently, a lower size limit of 31 µm has been proposed by deep-sea meiobenthologists in order to quantitatively retain even the smallest meiofaunal organisms (Giere, 2009). However this definition of meiofauna by sieve size raises some problems because juvenile forms of macrofauna are frequently included in the meiofauna, hence many authors have acknowledged this by distinguishing between *temporary* meiofauna (i.e. larvae and juvenile stages of species that, as adults, belong to the macrofauna) and *permanent* meiofauna (species belonging to the meiofauna during the whole of their life cycle) (McIntyre, 1969). For the scope of this work, meiofauna was defined as those metazoan fauna associated with estuarine sediments that pass through a 1000 µm sieve but are retained in a 38 µm sieve.

Meiofauna is a heterogeneous taxonomic and ecological group. Taxonomically, because the vast majority of the recognized animal phyla have meiobenthic representatives either their entire life cycle or just part of it (Higgins & Thiel, 1988; Vincx, 1996). Ecologically, because they occupy a broad variety of ecosystems ranging from mountain lakes to oceanic trenches and even in the same ecosystem they occupy totally different habitats. For instance, in estuaries, different meiofaunal communities occupy different habitats that go from mud to sand and from low to high salinities (Coull, 1999). This group is the most abundant and diverse metazoan group in aquatic sediments (Coull, 1999) and corresponds to *ca*. 60% of total metazoan abundance on Earth (Danovaro et al., 2010). Their spatial, temporal and vertical abundances are influenced by several biotic and abiotic and factors (Coull, 1999; Moodley et al., 2000; Giere, 2009). According to several authors (*e.g.* Higgins & Thiel, 1988; Soetaert et al., 1995) the variations in physical factors seem to explain better broad scale patterns in the abundance and distribution of meiofauna while biotic factors influenced by a complex factorial combination of attraction (*e.g.*, as a result of reproductive activities) or avoidance reactions (*e.g.*, predation) seem to regulate small-scale distribution at the centimeter scale (Li et al., 1997).

Biotic habitat factors tend to form a connected complex that greatly influences the structure of meiofauna communities with the activities of other fauna, such as disturbance, competition and predation, playing an important part (Giere, 2009) but, according to Moodley et al. (2000), it is the food availability that plays the lead role. For Coull (1999), the main abiotic factors that can explain meiofauna abundances are: salinity, temperature and sediment particle size. It has long been documented that the structure of the sediment is a major factor influencing meiofauna community structure (Warwick & Buchanan, 1970; Heip et al., 1985; Coull, 1988).

Indeed, when describing the habitats of meiofauna, grain size is a key factor since it directly defines spatial and structural conditions and indirectly defines the physical and chemical settings of the sediment (Giere, 2009). The sediment particle size is particularly important for vertical distribution. In general, the fauna burrows in sediments with a mean particle size of <125 µm, whereas sediment with larger mean grain sizes tend to have interstitial representatives (Coull, 1999). Vertical distribution of meiofauna has been intensively investigated in a wide variety of habitats, these studies (*e.g*. Huys et al., 1986; Escaravage et al., 1989; Adão, 2004) indicating that most meiofauna was restricted to the upper few centimeters in muddy sediments. Since the anoxic sediment zone is closer to the sediment surface in finer sediments, the fauna here is restricted to the very upper sediment layers because most meiofauna needs oxygen to metabolize. Thus in muddy estuarine sediments fauna is restricted to the narrow 2–3cm oxic zone, whereas in sandy sediments fauna may be distributed to depths of >10 cm (Coull, 1999). Other authors (*e.g*. Soetaert et al., 1995; Adão et al., 2009), focusing particularly on nematodes communities, sate that salinity regime in estuaries is the key factor determining the communities structure and controlling species composition, abundance and diversity. Thus, meiofaunal abundances vary greatly according to the habitat. The lowest abundance values are usually found in clean sandy sediments while the highest are found in organically enriched muddy sediments (Coull, 1999; Giere, 2009). Also, there tend to be decreased abundance and number of species as one move from the sea to freshwater (Coull, 1988).

According to McIntyre (1969), abundances range between 30 and 30,000 individuals per 10 cm^{-2}. If 1,000 to 2,000 individuals 10 cm^{-2} can be assumed to be an average value for all habitats, the meiofauna would exceed the macrofauna in abundance by two to threefold (Giere, 2009). Usually nematodes are the most abundant taxon comprising 60–90% of the total fauna and copepods are usually second at 10–40% (Coull, 1999; Moodley et al., 2000). The taxa Turbellaria, Oligochaeta, Polychaeta, Ostracoda, Mystacocarida, Halacaroidea, Hydrozoa, Nemertina, Entoprocta, Gastropoda, Aplacophora, Brachiopoda, Holothuroidea, Tunicata, Priapulida, Sipunculida, Gastrotricha, Gnathostomulida, Kinorhyncha, Loricifera and Tardigrada (the last five taxa are exclusively meiobenthic) usually comprise 5% or less of the total meiofauna (Vincx, 1996).

Meiofauna taxa are so diverse that there are only a few general trends in morphological adaptations (Giere, 2009). Its morphology, physiology and life history characteristics have evolved to exploit the interstitial matrix of marine soft sediments (Kennedy & Jacoby, 1999). The prime requisite to successfully explore the interstitial spaces of marine sediments is to be small, at least in one dimension (*e.g.*, body width). This requisite is mainly accomplished by reducing the number of cells while keeping the average cell size fairly constant thus leading to a simplification of body organization or to a loss of organs (Giere, 2009). The minute body sizes of meiofauna largely determine their reproduction and developmental modes. Meiobenthic animals have developed specialized approaches to sperm transfer, fertilization and development (Giere, 2009). For instance, unlike plankton, fish and benthic macroinvertebrates which have pelagic larvae that recruit from distant areas, meiofauna taxa have direct development with no larval stage. Meiofauna is confined to the substrate throughout their entire life cycle, with a generation time generally of less than one year and frequently with continuous reproductive periods (Austen & Widdicombe, 2006; Giere, 2009). Many meiobenthic animals have also developed specialized locomotive organs and characteristic modes of locomotion, often in conjunction with adhesion or elongation of the body and the reinforcement of external and internal body structure in order to protect them from the aggressions of a mobile habitat (Giere, 2009).

According to Coull (1999), in estuarine sediments meiofauna have two major ecological functions: i) facilitate of the biomineralization of organic material and enhance nutrient regeneration and ii) serve as food for a variety of higher trophic levels. Meiofauna can enhance transport of solutes in sediments and, thereby, increase the carbon mineralization in the upper sediment layers (Aller & Aller, 1992; Berg et al., 2000). Meiofauna also owns organic molecules and, because of their relatively short generation times, these molecules are rapidly returned to the system becoming part of the microbial loop where they are utilized by bacteria converted into dissolved organic carbon and used by higher trophic levels and/or remineralized for primary producers (Coull, 1999). Bioturbation caused by meiofauna enhances the sedimentary production of nitrogen gas and denitrification is a critical process that can alleviate the effects of excessive nitrogen availability in aquatic ecosystems subject to eutrophication (Bonaglia et al., 2014).

Meiofauna establishes an important link between smaller and larger organisms in benthic sediments mainly through trophic interactions (Piot, 2014). As a group, meiofauna consume a wide variety of food sources, including detritus, bacteria, diatoms and other small photoautotrophs, cyanophytes, ciliates and other meiofauna by predation or scavenging (Moens & Vincx, 1996). Meiofauna is also a very important nutritional source for organisms of higher trophic levels, which could not survive without them (Vincx, 1996; Coull, 1999), namely some commercially important fishes like flatfish and salmonids or for fish which are themselves food for commercial species (Gee, 1989). Danovaro et al. (2007) estimated that at Mediterranean sites studied in exclosure experiments, *ca*. 75% of the metazoan meiofauna in soft bottoms is channeled to higher trophic levels with Polychaetes and Nematodes providing the major contribution to benthic energy transfers.

In spite the research done in the last decades has showed that meiofaunal communities play a very important role in marine sediments; indirectly, by processes such as bioturbation and the stimulation of bacterial metabolism and, directly, as food sources for organisms in higher trophic levels (Vincx, 1996). Recent research indicated that the function of meiofauna in marine benthic systems seems to be much more complex than previously supposed, and requires investigation to clarify its ecological importance in the benthic domain (Balsamo et al., 2012).

Free-living marine nematodes: abundance, diversity, horizontal and vertical distribution and feeding ecology

Abundance, *i.e.*, the number of individuals in a given area (or volume), is widely used as an important characteristic of biological communities to infer relationships between the community and its environment (Moens et al., 2013). In meiofauna samples the number of species of nematodes often exceeds that of all the other groups put together by an order of magnitude (Giere, 2009). Moreover, marine nematodes are the most abundant and diverse metazoans in littoral, estuarine, coastal and oceanic sediments (Higgins & Thiel, 1988; Heip et al., 1982; Bouwman, 1983; Heip et al., 1985; Soetaert et al., 1995). Bouwman (1983) attributed nematode dominance in estuarine sediments to three main factors: (i) their burrowing capacity, in combination with their small and slender shape, allowing the occupation of interstitial spaces in coarse grained sediments as well as the invasion of soft sediments; (ii) their tolerance, as a taxon, to a variety of environmental stresses; (iii) well-developed sensory organs together with the diversification in buccal structures, enabling nematodes to exploit a broad range of food items present in the benthos.

Nematodes have also been flagged as one of the most diverse group on the marine ecosystems (Platt et al., 1980). For nematodes, the most widely used diversity descriptor is species or genus richness (Moens et al., 2013). Complementary to species or genus richness, many other diversity indices (*e.g.* Simpson, Shannon-Wiener, Pielou´s evenness) and richness estimators (*e.g.* Chao, Ugland-Gray-Ellingsen) have been proposed and are regularly used to describe nematode assemblages (Clarke & Warwick, 2001). Current species richness estimates range between 10,000 and >1,000,000 species (Mokievsky & Azovsky 2002, Lambshead & Boucher, 2003). Appeltans et al., (2012) suggest that about 85% of the marine nematode species are still unknown to science.

Nematodes show an aggregated and patchy distribution in virtually all marine habitats (Hogue, 1982; Decho & Fleeger, 1988; Eckman & Thistle, 1988; Hodda, 1990; Li et al., 1997; Gallucci et al., 2009) due to complex interactions between biotic and abiotic factors (Coull, 1999; Moens et al., 2013). According to several authors (*e.g*. Hogue & Miller, 1981; DePatra & Levin, 1989), at the small-scale, effects of sediment microtopography, like the aggregation of food sources and/or the reduction in hydrodynamics, is one of the most important factors related to changes in the abundance and composition of nematode assemblages. Nematodes are highly influenced by small-scale patches of organic matter and disturbance that create microhabitats in space and time that harbor distinct nematode assemblages in different successional stages, allowing the coexistence of many different species at very small scales (*e.g*., Gallucci et al. 2009). The higher the degree of habitat microstructure, the richer is the nematode assemblage and that’s why, usually, heterogeneous fine sands in shallow sea bottoms with a rich food supply and an interstitial system that provides enough solute and oxygen transport, harbor the highest number of species

(Giere, 2009). At the large-scale, nematode distribution patterns and assemblage structures have been linked mainly to physical-chemical factors like sediment grain size (*e.g*. Steyaert et al., 2003) and salinity (*e.g*. Adão et al., 2009). The influence of sediment size is shown by fact that muddy and sandy habitats harbor different assemblages with distinct characteristics: nematodes from sandy habitats tend to be more slender as they have to move through the interstitial apertures, whereas nematodes from muddy habitats are generally more robust for burrowing through the sediment (Tita et al., 1999).

Salinity gradient has been positively related to nematode diversity at the genus level (Soetaert et al., 1995). While most freshwater water species are restricted to salinities lower than 10, several marine species can be found both at almost freshwater conditions and at salinities up to 35 or higher (Heip et al., 1985). In general, brackish waters are characterized by a lower number of species than either marine or freshwater regions (Heip et al., 1985). This may be due to high variability in salinity at this portion of estuaries with less species being able to tolerate such conditions. Austen & Warwick (1989) suggested salinity variation affects the selective, specialist feeding types by interfering with food sources thus reducing community diversity. Tietjen & Lee (1972, 1977) reported that the generation time of two nematode species were increased both by reduced and increased salinity indicating that salinity variation also interferes with normal life cycle processes.

At the vertical dimension, changes in nematode assemblage structure occur at the scale of few centimeters or even a few millimeters, due to a more pronounced change in environmental factors, such as food resources and oxygen availability (Heip et al., 1985; Steyaert et al., 2003). Sediment oxygen concentration decreases with depth down to the limit where anaerobic processes start to dominate, and because sulfide ions produced in the anoxic layer are toxic to most aerobic species, the transition zone between the oxygenated layers and the redox potential discontinuity (zone between oxygenated and reduced conditions) layer is the lower limit of depth distribution for many species (Moens et al., 2013). These abrupt changes along the vertical dimension cause significant decreases in nematode densities, numbers of species and changes in species composition (Joint et al., 1982; Steyaert et al., 2003; Adão, 2004). Nematodes assemblages living in the top more oxygenated layers are, in fact, more abundant, species rich and less variable, in terms of species presence/absence and relative abundances, than assemblages living in the deeper reduced layers (Vieira & Fonseca, 2013). In intertidal areas, nematode vertical distribution may also be controlled by the quick changes in abiotic conditions during low tides. Factors such as temperature, wave action, currents, organic input, percolation of interstitial water, light intensity, compaction of the sediment and others will change periodically with the tidal cycle forcing benthic organisms adapt to an unstable environment or to migrate deeper into sediment layers (Steyaert et al., 2001). It is the case of many nematodes that are sensitive to low water content in the sediment (Jansson, 1968) and migrate to deeper layers during low tide. As the tide comes in and the conditions are reestablished, they return to the surface (McLachlan et al. 1977; Steyaert et al. 2001). Nevertheless, other species have an opposite reaction migrating upward during low tide and downward during submersion (Steyaert et al. 2001). This might happen due to increased diatom production and biomass build-up at the sediment surface during tidal exposure (Guarini et al. 1997) or to escape predacious species (Gallucci et al., 2005).

The nematode assemblage is generally studied in terms of density, diversity, assemblage structure, life history strategies, body size, biological trait analysis and trophic guilds. The Index of Trophic Diversity (ITD) (Heip et al., 1985) is one of the most commonly used indices in ecological studies of nematode communities (Balsamo et al., 2012) because the ecological value of nematodes is related not only to their notable quantitative importance in the benthic domain, but also to their pivotal role within the food webs of the aquatic ecosystems (Platt et al., 1980).

Free-living aquatic nematodes may feed on diverse array of resources, such as bacteria and Archaea, protists, fungi, particulate and dissolved organic matter, and as predators prey on metazoans, including other nematodes (Moens et al., 2013). The vast majority of marine nematodes have, traditionally, been assigned to feeding types or guilds based on the morphology of their stoma. Wieser (1953) classified nematode feeding groups and types according to their buccal cavity structure proposing a classification with four feeding types with a primary subdivision between nematodes with and without “buccal armature”. Buccal armature refers to the presence of a tooth or teeth, onchia, denticles, mandibles or other sclerotized structures (Moens et al., 2013). Within the first group are the nematodes with no buccal armature (groups 1A and 1B). They are both called deposit feeders, but distinguished from each other based on the size of the buccal cavity. 1A nematodes are referred to as "selective deposit feeders" and are characterized by small to minute mouth openings, which only allow ingestion of very small, bacteria-sized food particle. 1B nematodes are the "non-selective deposit feeders" and have bigger buccal cavities, enabling them to exploit a wider array of differently sized particles and, occasionally, other metazoans (Moens & Vincx, 1997). The second group encompasses the nematodes with buccal armature (groups 2A and 2B). Group 2A nematodes are called "epistratum feeders" and are characterized by the presence of a tooth, denticles or other sclerotized structures in the stoma. These mouthparts can be used to either scrape off bacteria or microalgae from a substratum, such as a sand grain, or to damage the target food cell to suck it empty. The latter can be done by piercing (the nematode sucks the food particle to its mouth and then partly everts its tooth to puncture it), or by cracking (the food particle is partly taken into the mouth, opened with a tooth, emptied and subsequently discarded) (Jensen, 1982; Moens & Vincx, 1997). Group 2B contains the "predator" nematodes. The nematodes belonging to this group are often large with big mouth openings, equipped with sclerotized structures like teeth, onchia and mandibles. Feeding mechanisms include piercing and emptying of a prey, tearing a prey open or even ingesting the entire prey (Moens & Vincx 1997, Fonseca & Gallucci 2008).

Although Wieser (1953) has the merit of being the first who found a relation between community structure and trophic guilds expressed in the cuticular armatures of the nematode's buccal cavity, posterior studies (*e.g*. Jensen, 1987; Moens & Vincx, 1997; Moens et al., 2004) suggest that the reduction of huge species diversity into four feeding types is likely to underestimate the true functional complexity of nematode communities. Wieser trophic classification scheme present some disadvantages: in one hand it does not take into account feeding selectivity or flexibility (Moens et al., 2004) and, on the other hand, the variability of buccal structures also depends on ontogenetic age (Lorenzen, 2000). Several changes to Wieser (1953) scheme have been suggested. Jensen (1987) proposed two fundamental changes: i) selective and non-selective deposit feeders groups were pooled into a single category of "deposit feeders"; and ii) a discrimination between real predators and scavengers among the predators/omnivores. Moens & Vincx (1997), proposed a modified feeding-type classification based on dedicated observations of the feeding behavior of a variety of estuarine nematodes, covering all feeding types proposed by Wieser (1953) and Jensen (1987). Moens & Vincx (1997) re-instated the basically mouth-size based division among deposit feeders, but did not link it to a different level of selectivity and selective deposit feeders were now labeled "microvores" and the non-selective deposit feeders became "deposit feeders". Currently, there is substantial evidence that, due to their restricted capacity for proteolytic nutrient digestion, nematodes are mostly selective feeders (Giere, 2009). Nevertheless, no matter the feeding-type classification chosen and even though the combination between trophic diversity and species richness can provide a good measure of environmental distress (Heip et al., 1985), traditional feeding type classifications of nematodes based on buccal morphology can be misleading and should be combined with empirical information for reliable conclusions (Vafeiadou et al., 2014). As nematodes have diverse feeding behaviors they play different roles in food webs. The increase, reduction or absence of a feeding-type can function as an important indicator of ecosystem process deregulation.

Nematodes trophic diversity is just one, among various, criteria that can be used as a useful tool for monitoring environmental impacts in an estuarine ecosystem, namely the ones caused by accidental or intentional introduction of an allochthonous species.

Biological invasions and non-indigenous species

The biosphere is experiencing unprecedented modifications as a result of human activities. Some of the most determinants changes in biodiversity at the global scale include, but are not limited to, land use change, climate change, altered hydrologic cycles and biological invasions (Sala et al., 2000; Hobbs & Harris, 2001; Ellis, 2011). A long-term view suggests that on all spatial scale, species ranges changes occur constantly and are an important force of natural communities structures (Lodge, 1993) but the accidental or voluntary redistribution of species by humans is now occurring at a pace that is impairing ecosystem services and threatens human health (Hassan et al., 2005; Pyšek & Richardson, 2011; Simberloff et al., 2013).

“Non-indigenous”, “non-native”, “alien” or “exotic” are frequently used as synonyms, referring to species that do not occur naturally in a certain area, *i.e.*, that are established outside of their native range due to an accidental or intentional transport to non-contiguous regions. On the contrary, “native” or “indigenous” refers to a species that occurs naturally in an area, i.e., whose dispersal has occurred independently of human translocation. (Manchester & Bullock, 2000; IUCN, 2002; Simberloff et al., 2012).

In their home ranges, species live in balance with their local native environment and populations are controlled by ecosystem interactions such as predation, parasitism and disease (Otero et al., 2013). However, once arriving at a new environment, they may become established and invasive if certain factors like the absence of natural enemies (deRivera et al., 2005) and the possession of certain biological traits such as wide environmental tolerance and high reproductive output are present (Angert et al., 2011). Once non-indigenous species become established in a new environment they have the capability of transforming local biological community interactions diminishing the size of native populations, modifying significantly the community structure, ecosystem function and even causing extinctions (Ruiz et al., 1997).

Nevertheless, according to some authors (*e.g.* Bax et al., 2003; Rodriguez, 2006; Vellend et al., 2007), non-indigenous species can, sometimes, have positive effects supporting native species through mechanisms that include habitat modification, trophic subsidy, pollination, competitive release, predatory release and they can also promote evolutionary diversification via increased genetic differentiation among populations of both non-indigenous and native species. In the particular case of non-indigenous marine species they can also act as improvement of aesthetic values, the creation of new economic activities (*e.g.* fisheries and aquaculture) and knowledge gained on ecosystem processes and resource dynamics and interactions (Bax et al., 2003).

Humans have greatly assisted the spread of aquatic invasive species through intentional stocking, aquarium releases, canal construction and international shipping (Rahel, 2007). Estuaries are among the most invaded and altered ecosystems by non-indigenous species (Ruiz, 1997). With the increased use of water courses in continental and maritime coast also increased intentional and accidental introductions of non-indigenous species mainly through activities such as navigation (Carlton & Geller, 1993) and aquaculture (Hulme et al., 2008). The impacts of non-indigenous species in marine environments are still greatly understudied and have received relatively little attention when compared to terrestrial and freshwater habitats (Ruiz et al., 1997; Lowe et al., 2000). Unfortunately the marine invasive species problem is worsening and it's utterly necessary that environmental, social and economic risks (still largely ignored) are taken into account by governments and environmental interest groups (Bax et al., 2003).

Some species introduced in aquatic ecosystems may rapidly form self-sustaining populations and thus pose major threats to the invaded communities (Kolar & Lodge, 2001). Bivalves are one of the most invasive groups, as many species can rapidly occur at remarkably high densities, accounting for the major proportion of the benthic faunal biomass (Sousa et al., 2009). Once well-established and widespread, introduced species are difficult to eradicate (Wasson et al., 2001), and to be able manage this threat it is necessary to have a strong understanding of how non-indigenous species can affect local species and communities dynamics. Moreover, the same species might have distinct impacts when introduced into different locations and although there are lists of the worst invasive species, impacts are always difficult to predict.

Ruditapes philippinarum: distribution, habitat, environmental and ecological impacts

R. philippinarum is a native species of the subtropical to low boreal western Pacific (Humphreys et al., 2007). Natural populations are distributed in the Philippines, South China and East China Seas, Yellow Sea, Sea of Japan, Sea of Okhotsk and around the Southern Kuril Islands (Goulletquer, 1997). It is currently widely distributed along the

Pacific coast of America, the Atlantic coast of Europe, and it also occurs in the Adriatic and Aegean seas (figure 1). *R. philippinarum* has been introduced and established viable populations in new areas outside its original range due to accidental introduction with oyster seed to North America and Canada and intentional introduction as broodstock in several European countries including France, Spain, Italy and Portugal (Goulletquer, 1997; Jensen et al., 2004, 2005; Campos & Cachola, 2006). This premeditated introduction was due to overfishing and irregular yields of the native European grooved carpet shell (*Ruditapes decussatus*) (Goulletquer, 2005).

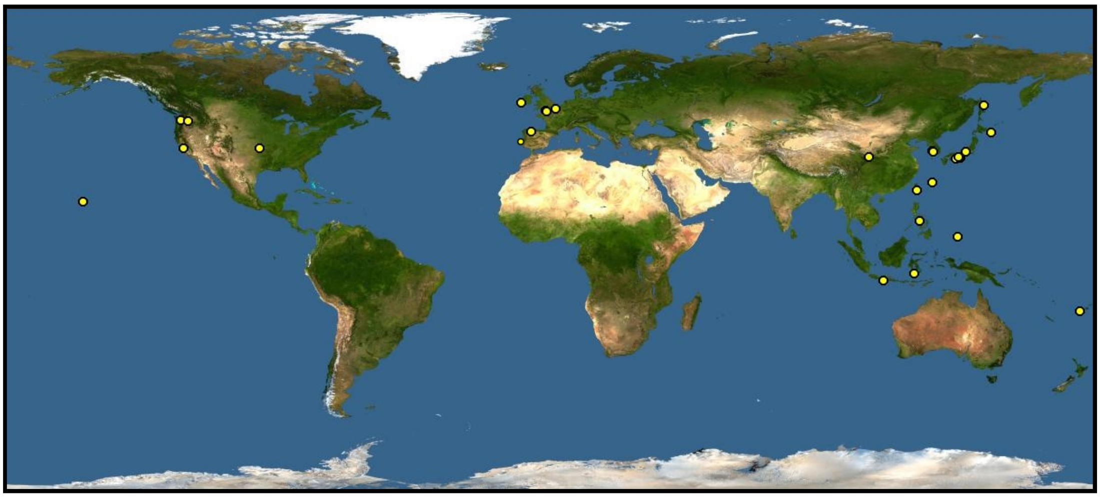

Figure 1 - *Ruditapes philippinarum* (yellow dots) global distribution (adapted from cabi.org).

R. philippinarum is a euryhaline bivalve widely distributed in intertidal zones with a 16-36 salinity range with an optimal interval between 20 and 26. It is generally found in estuaries and lagoons, on sandy and muddy bottoms, from the surface to a few meters depth (Kim et al., 2001) and it can reach concentrations over 5000 ind/m^2 (Lee, 1996). Humphreys et al. (2007) suggests that *R. philippinarum* is most successful in eutrophic conditions such as those that can occur in coastal lagoons and similar environments, but the species is not restricted to such locations.

R. philippinarum is considered a high efficiency filter feeder (Nakamura, 2001) that consumes plankton and micro-organisms that are carried by currents along the bottom. Watanabe et al., (2009) stated that specimens larger than 5 mm in shell length assimilated mainly benthic particulate organic matter (POM), while individuals smaller than 5 mm assimilated mostly benthic and pelagic POM. The fact that suspension feeders have relatively larger gills than palps for pumping, whereas deposit feeders have relatively larger palps than gills for sorting and that the relative sizes of the gills and palps are related to changes in local food conditions it raises the possibility of diet shifts in relation to body size (Compton et al., 2008). In fact Suh & Shin (2013) data reveals that bigger clams are more prone to feed on a larger variety of food sources while smaller clams have to be more selective. Nevertheless controversy remains regarding the relative contribution of benthic or pelagic POM to the diets of *R. philippinarum* specimens of varying sizes (Dang et al., 2009). According to Nakamura (2001) *R. philippinarum* shows a higher efficiency to filter small particles (picocyanobacteria to autotrophic nanoplankton, 2–8 µm long) when compared to *R. decussatus*, which might be one of the causes for the disappearance of indigenous *R. decussatus* in Northern Adriatic lagoons.

In many shallow-water ecosystems like rivers, estuaries, and coastal marine waters, bivalve grazing rates are greater than adventive losses, implying that bivalves may affect the structure and function of these ecosystems (Strayer et al., 1999). At high densities (over 2000 adult individual m^{-2}), *R. philippinarum* can affect nutrient dynamics (Bartoli et al., 2001) and modify the abundance of zooplankton (Sorokin et al., 1999).

Ruditapes philippinarum in Portugal

R. philippinarum presence in Portugal is now known for more than two decades. It has been recorded in several estuaries (*e.g.* the Tagus and Sado River), lagoon systems (*e.g.* Ria Formosa and Ria de Aveiro) and coastal lagoons (*e.g.* Lagoa de Albufeira) (Campos & Cachola, 2006; Gaspar, 2010).

Despite the generally low abundance of this species in the Portuguese colonized systems, its populations have increased greatly along the last years and it is currently the dominant benthic species in some areas of the Tagus estuary, mainly in shallow bays with extensive intertidal areas. The increase in abundance of *R. philippinarum* was simultaneous to a strong reduction of the native congeneric species *R. decussatus* (Gaspar, 2010; Garaulet, 2011; Chainho et al., 2015).

Estuaries

The most widely accepted definition of an estuary was proposed by Pritchard (1967) and, according to his definition, an estuary is “a semi-enclosed coastal body of water which has a free connection with the open sea and within which ocean water is measurably diluted with freshwater derived from land drainage”.

Estuaries are formed at the mouth of rivers in the narrow boundary zone between sea and land (Dyer, 1998). Estuaries and the nearby coastal zones are characterized by steep gradients in chemical, physical and biological features (Ysebaert et al., 1993) and are, thereby, highly dynamic environments. Mainly influenced by the hydrological regime, there is a biotic change along the estuarine complex-gradient that results in major spatial differences. However, these spatial patterns can change over time, mainly in poikilohaline estuaries, characterized by strong seasonal changes promoted by freshwater discharges (Boesch, 1977; Chainho et al., 2006).

Estuarine areas are considered transitional environments and are characterized by a unique biodiversity that places them among the most productive ecosystems (Kennish, 2002; Dolbeth et al., 2003). Their importance is recognized worldwide, for providing essential ecological functions like decomposition, nutrient cycling and water flux. Estuaries are also important as habitat, protection and food source for large number of resident and migratory species. They’re also essential in shoreline protection, fisheries resources, navigation routes, harbors and for recreational purposes (Kennish, 2002). But one of the most important characteristics of estuaries is that they form a mosaic of inter-linked habitats that shouldn’t be considered in isolation (Morrisey et al., 2003) because each one is of particular value for the different species that use it. The assessment of the importance, dynamics and functions of each estuarine habitat, as well as their ecological connectivity, is still poorly understood (Elliott & Hemingway, 2002).

The unique physical and chemical attributes of estuaries relate primarily to the large volumes of freshwater and sediments delivered to the sea by rivers. The mixing zone for freshwater and seawater within the estuary can be exceptionally complex, affected by the volume and rate of discharge of fresh water from the river, the amount and grain size of sediments in the river, the topography of the coastline, the tidal range, and the strength and direction of prevailing wind and waves (Dyer, 1997).

The Tagus estuary

The Tagus estuary, with an area of 320 km^2 it is the largest estuary in Portugal and one of the largest in Europe. Morphologically the Tagus estuary can be divided into three main areas: a straight narrow and deep W-E oriented channel with about 16 km long and 2 km wide, with maximum depths of about 45 m; an inner bay with 25 km long and 15 km wide SW-NE oriented with depths between 5 and 10 m and; an upper shallow estuary with an area of 100 km^2 with large mudflats and salt marshes separated by shallow channels (Braunschweig et al., 2003). Intertidal mudflats encompass 40% of the estuarine area (Cabral & Costa 1999).

This estuary is a semi-diurnal mesotidal estuary, with tidal range varying from 1 m during neap tides up to almost 4 m in the spring tides. The tide propagates up to almost 80 km upstream and the estuary mean resident time is 25 days. The Tagus river flow is the main source of freshwater with a flow typically between 50 to 2000 m^3 s^{-1}, showing a strong seasonality and also controlled by dam releases. The Sorraia and the Trancão rivers are the second and third main estuarine tributaries with mean discharges of 39 and 6 m^3 s^{-1}, respectively (Braunschweig et al., 2003). Salinity varies from freshwater (0.0), 50 km upstream from the mouth, to marine (36.0) at the mouth of the estuary. The water temperature ranges from 8 to 26°C (Cabral & Costa 1999).

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|GENERAL AIMS

Invasive species are considered today one of the key drivers of biodiversity loss. At first, many of the voluntary introductions can be seen as an economic boon, but in the long term the social and economic impacts of invasive species can be devastating. Together with climate change the impacts of each of these drivers of change are compounded, and interactions between them present even greater challenges to field conservationists as well as policy makers. Awareness and mitigation of both factors should underpin all biodiversity management planning and policy.

The main aim of this research was to investigate the spatial distribution patterns of meiofauna assemblages along the Tagus estuary with a special focus on the ecology of free-living marine nematodes and relate it with the spatial distribution and abundance of the non-indigenous *bivalve R. philippinarum*.

The major questions the served as a baseline for this study were: i) is the spatial distribution and abundance of the non-indigenous bivalve *R. philippinarum* related to the spatial distribution patterns of the meiofauna assemblages? ii) are there differences in density, genera and trophic composition of the nematode communities associated to R. *philippinarum abundances*? iii) do environmental variables override the effects of *R. philippinarum* over the meiofauna/nematodes communities? The following null hypothesis were tested to answer these questions: a) there are no differences in spatial patterns of the meiofauna assemblage abundance associated to different abundances of the non-indigenous bivalve *R. philippinarum* and b) there are be no differences in spatial patterns of the nematode assemblage abundance, genera and trophic composition associated to different abundances of the non-indigenous bivalve *R. philippinarum*.

|SCIENTIFIC ARTICLE

Effects of the non-indigenous bivalve *Ruditapes philippinarum* on meiofaunal communities of the Tagus estuary

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Keywords

Impacts of invasive species / nematodes / non-indigenous species / estuarine gradient / benthic assessment tools

Abstract

The use of benthic assessment tools based on meiofauna communities is a recent but promising research area since macrofauna has been the most common biological indicator use in the assessment and monitoring of natural and anthropogenic disturbances in aquatic ecosystems and the numerous advantages of meiofaunal organisms over macrofauna have been largely ignored.

This study aims to investigate the response of meiofauna assemblages, focus in nematodes genera composition, to the presence of the non-indigenous bivalve *Ruditapes philippinarum* native to the western Pacific Ocean and first introduced in Europe between 1972 and 1975. Density, diversity and trophic composition patterns were examined along the Tagus estuary gradient to determine: i) the spatial distribution patterns of meiofauna communities, ii) the spatial distribution patterns of nematode assemblages and iii) the most important environmental variables influencing the meiofauna and nematode assemblages. The following hypotheses were tested: i) Is the spatial distribution and abundance of the non-indigenous bivalve *R. philippinarum* related to the spatial distribution patterns of the meiofauna assemblages? ii) Are there differences in density, genera and trophic composition of the nematode communities associated to R*. philippinarum abundances*? iii) Do environmental variables override the effects of *R. philippinarum* over the meiofauna/nematodes communities? The number of meiofaunal taxa recorded in this study can be considered high but the densities of meiofauna are average when compared the densities recorded in other studies. The nematodes taxon presented the highest density (>80%) in all sampling sections. The obtained results showed that even though the distribution pattern of nematodes was mainly structured by distinct environmental factors like sediment grain size and salinity *R. philippinarum* densities are also important.

Introduction

An invasive species is a non-indigenous species that has a tendency to spread and may cause damage to the environment, human economy and/or human health. Invasive species threaten native biodiversity by negatively impacting on local biological community interactions, diminishing the size of native populations, modify significantly the community structure, ecosystem function and even causing extinctions (Ruiz et al., 1997). Even though biological invasions may be natural and necessary for the persistence of life, namely through species range expansion, the human mediated introduction and spread of invasive species has been identified as a major ecological threat in coastal marine communities (Cohen & Carlton, 1998; Occhipinti-Ambrogi, 2007; Claudet & Fraschetti, 2010).

Largely due to overfishing and irregular yields of the European native grooved carpet shell clam (*Ruditapes decussatus*), the congeneric Manila clam (*Ruditapes philippinarum*), native to the western Pacific Ocean, has been one of the most widely introduced species for aquaculture purposes in Europe (Goulletquer, 1997; Jensen et al., 2004). It was first introduced between 1972 and 1975 in France and later in England, Spain and Italy (Flassch & Leborgne, 1992; Humphreys, 2010). In Portugal its presence is known for more than two decades and it has been registered in several estuaries (*e.g*. the Tagus and Sado River), lagoon systems (*e.g*. Ria Formosa and Ria de Aveiro) and coastal lagoons (*e.g*. Lagoa de Albufeira) (Campos & Cachola, 2006; Gaspar, 2010).

Although some research has been done relating the impacts of bivalve introduction on macrofauna communities, studies of the impact of bivalve on meiofaunal communities have been scarce and restricted to the harvest effect (*e.g*. Fiordelmondo et al., 2003; Mistri et al., 2004 or Lin et al., 2007). For reasons of convenience, most biological indicators have traditionally been large visible organisms that can readily be counted and identified and meiofauna, due to its small size, has rarely been included (Kennedy & Jacoby, 1999). However, more recently, meiofauna features such as their high abundance, rapid generation times and absence of a planktonic phase together with new technologies and tools, such as electronic identification keys, molecular approaches and the creation of new indices helped to increase the awareness of meiofauna as a tool in ecological studies (Kennedy & Jacoby, 1999; Schratzberger et al., 2000; Austen & Widdicombe, 2006) namely monitoring the impact of aquaculture activities (Mazzola et al., 2000; Mirto et al., 2000). Nematodes in particular have been pointed out as potential indicators of anthropogenic disturbance in aquatic ecosystems (*e.g*. Coull & Chandler, 1992; Steyaert et al., 2007; Moreno et al., 2008). The inclusion of information regarding their functional traits (*e.g*. trophic structure and life strategy) can provide critical information on the functioning of ecosystems (Norling et al., 2007; Danovaro et al., 2008). Sediment structure, chemistry, disturbance and availability of food, such as bacteria and microphytobenthos, are closely linked to nematode assemblage composition and distribution patterns (Heip et al., 1985; Moens et al., 2005; Giere, 2009), through the changes in density, diversity, structure and functioning it is possible to detect changes alterations in the system. Therefore, characterizing the distribution patterns of meiofauna and nematodes assemblages has become a useful biological tool to detect disturbances and environmental change. Benthic organisms are generally influenced by complex and interacting physical and biological processes, leading to variation in their distribution at different spatial and temporal scales and identifying these temporal and spatial distribution patterns is an essential step towards understanding the processes structuring ecological communities (Underwood & Chapman, 1996; Gallucci et al., 2009).

The meiofauna communities are reasonably well characterized around the world, with studies ranging from the deep-sea floor to alpine lakes or from tropical reefs to polar sea ice (Giere, 2009). In Europe, studies on meiobenthic estuarine communities mostly include the more northerly estuarine ecosystems (*e.g*. Warwick & Gee, 1984; Heip et al., 1985; Li & Vincx, 1993; Smol et al., 1994; Soetaert et al., 1994; Steyaert et al., 2003; Ferrero et al., 2008; Rzeznik-Orignac et al., 2003). In southern Europe, particularly in the Iberian Peninsula, there is still a notorious lack of information on both spatial and temporal distribution of meiofauna and free-living nematodes in estuarine environments. Although in Portugal there are several studies of meiofauna and nematodes distribution at different spatial and temporal scales of estuarine environments (*e.g*. Adão et al., 2009; Alves et al., 2009; Materatski et al., 2015) the amount of information available on Tagus estuary meiofauna is low, consisting mostly of small scale studies (*e.g*. Soetaert et al., 1995; Franco et al., 2008) and until now there are no studies characterizing the distribution patterns of meiofauna or nematodes communities that comprise all estuary area.

Considering the interaction between individuals or species in a heterogeneous environment, this study aimed at: i) ascertaining the spatial distribution patterns of the density and composition of the meiofaunal communities with special focus on nematodes assemblages along the Tagus estuary and ii) to relate it with the density of the non-indigenous bivalve *R. philippinarum*. Those objectives were investigated by testing the following hypotheses: i) are there differences in the density and composition of meiofauna communities between areas with and without the presence of the non-indigenous bivalve *R. philippinarum*?; ii) are there differences in density, genera and trophic composition of the nematode communities between areas with and without the presence of the non-indigenous bivalve *R. philippinarum?* and iii) are environmental variables more important for the structure of these communities than the presence of *R. philippinarum*?

Study area and sampling design

This study took place at the Tagus estuary, located on the Western Coast of Portugal (38◦44′ N, 9◦08′ W) with an area of ca. 320 km2 and approximately 50 km long. Its width varies from 15 km, at the upper area, to 2 km near the mouth (Gaudêncio & Cabral, 2007). According to the North American National Oceanic and Atmospheric Administration (NOAA) it is classified as a mesotidal estuary, with semi-diurnal tides ranging from 0.4 m at neap tide to 4.1 m at spring tide (Duarte et al., 2013). Approximately 40% of the estuarine area is composed of intertidal mudflats. In the upper and middle estuary the prevalent sediment is muddy sand and in the low estuary and adjoining coastal area the dominant sediment is sand (Cabral & Costa 1999).

The estuary is characterized by profound hydromorphological change (Chainho et al., 2008) and its hydrography is modulated by the tidal propagation and fluvial discharge from the major tributaries: Tagus, Sorraia and Trancão Rivers (Vaz et al., 2011). The Tagus river has an annual mean flow is 400 m^3s^{-1}, with monthly discharges that may vary from 100 to 2200 m^3s^{-1} (Cabral et al., 2001; Chainho et al., 2008). Seawater enters the estuary through a deep narrow, inlet channel and tidal influence reaches 80 km inland from Lisbon (Duarte et al., 2013). Together, these interconnected forces induce the appearance of sharp gradients of salinity inside the estuary with the formation of three distinct regions: a marine region (lower estuary), a mixing region (middle estuary) and a region where the freshwater inflow dominates (upper estuary) (Vaz et al., 2011). Within the estuary, salinity varies from freshwater (0.0), 50 km upstream from the mouth, to marine (36.0) at the mouth of the estuary (Cabral et al., 2001). Unlike smaller estuaries (*e.g*. Mondego and Mira) in regular years there's almost no difference between summer and winter salinity values registered along the estuary (Chainho et al., 2008). The water temperature ranges from 8°C to 26°C (Cabral et al., 2001). Major human pressures are related to the discharge of effluents from agricultural, industrial, and urban sources, as well as the location of one of the major national commercial harbors. It is adversely affected mainly by the inflow of effluents from about 2.5 million Greater Lisbon inhabitants (Chainho et al., 2010).

Meiofaunal community samples were collected to determine the density, diversity and trophic composition of the meiofaunal communities. Sampling surveys were conducted during May 2013 as part of the project “Identification of the effects of the Manila clam on the biological communities in the Tagus estuary; Impacts of the Manila clam harvesting”. Meiofauna samples were collected at 40 sampling stations distributed along the Tagus estuary in the known occurrence area of the Manila clam and grouped in four different sections according to previous studies of Tagus estuary hydromorphological characteristics: “Upstream” comprehending the fifteen stations closest to the river source and majorly influenced by fresh water inputs; “Intermediate” that included the eight stations closer to the Vasco da Gama bridge with a mixed influence from freshwater and marine waters; “Bay” section which encompassed nine station along Almada, Seixal, Barreiro, Moita and Montijo bay was designed because these bay areas suffer little or no influence from Tagus main tributary. “Downstream” grouping the eight closest stations to the estuary mouth area and suffering major influence from marine water inputs (figure 2).

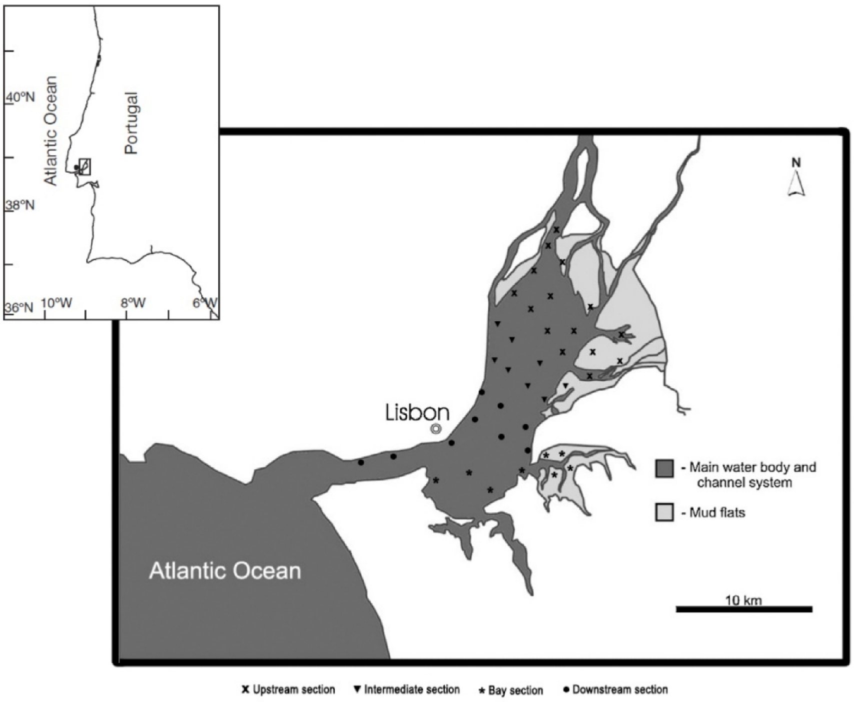

Figure 2 – Tagus estuary (Portugal): indication of meiofauna sampling sections and stations.

Sampling and laboratory treatment

Environmental data

Bottom water parameters were measured at each sampling station, in situ with an YSI Data Sonde Survey 4: salinity (Practical Salinity Scale), temperature (◦C) and dissolved oxygen (DO) (mg L^{-1}). Approximately 100g of sediment were collected at each sampling station to determine the total organic content and grain size. Sediment total organic content (OM) was determined as the difference between the weights of each sample after oven drying at 60 ◦C for 72 h followed by combustion at 450 ◦C for 8 h, and was expressed as the percentage of the total weight. Grain size was determined by dry mechanical separation through a column of sieves of different mesh sizes, corresponding to the five classes described by Brown & McLachlan (1990): (a) gravel (>2 mm), (b) coarse sand (0.500–2.000 mm), (c) mean sand (0.250–0.500 mm), (d) fine sand (0.063–0.250 mm), and (e) silt and clay (<0.063 mm). The relative content of the different grain size fractions was expressed as a percentage of the total sample weight.

Biological Data

Meiofauna samples were collected at each sampling station by forcing a 3.5cm inner diameter transparent Plexiglas tube 3cm into the bottom sediment collected by van Veen grab with a sampling area of 0.1 m^2. All samples were preserved in 4% buffered formalin solution. At the laboratory, all meiofaunal fixed samples were rinsed under a gentle jet of fresh water over a 1000 µm sieve to exclude macrofauna, followed by a sieving using a 38 µm mesh. The retained 38-1000 µm fraction was washed and centrifuged three times with Ludox HS40 (specific density 1.18 g cm^{-3}). The supernatant of each washing cycle was again collected on a 38 µm sieve. Samples were preserved in a buffered 4% formalin solution and stained with Rose Bengal after extraction. All metazoan meiobenthic organisms were counted and identified to the lowest possible taxonomic level following Higgins & Thiel (1988) and Giere (2009) under a stereomicroscope (40X magnification) and the density (individuals per 10 cm^{-2}) of each taxon was quantified.

For identification, 100-120 nematodes (or the total number of individuals in samples with less than 100 nematodes), were randomly picked from each replicate, transferred through a graded series of glycerol-ethanol solutions, stored in anhydrous glycerol, and mounted on slides (Vincx, 1996) and identified. All nematodes were identified to genus level using a microscope fitted with a 100X oil immersion objective and based on the pictorial keys of Platt & Warwick (1983, 1988), Warwick et al. (1998) and NeMys (Vanaverbeke et al., 2014). All individuals identified were grouped into four feeding-type groups: selective deposit feeders (1A), non-selective deposit feeders (1B), epigrowth feeders (2A) and predators/scavengers (2B) according to the Wieser (1953) classification.

R. philippinarum samples were collected using a mechanical shellfish dredge with an opening with 60 cm width x 30 cm height and a net mesh size of 3 x 3 cm. A fishing boat was used to conduct 30 seconds tows at an average commercial speed of 1.2 knots at each sampling station. The number of individuals was counted and density was determined as the number of individuals per 30 seconds tow.

Data analysis

Data was analyzed in order to (a) determine spatial distribution patterns, density and composition of the meiofauna and, particularly, the nematodes assemblages along the Tagus estuary; (b) to identify the environmental variables related with the spatial patterns obtained and (c) to relate the meiofauna and nematodes density with the spatial distribution pattern of the non-indigenous bivalve *R. philippinarum*.

The statistical analyses of biological and environmental data were performed using the PRIMER v6 software package (Clarke & Warwick, 2001) with the PERMANOVA add-on package (Anderson et al., 2008).

Environmental variables

A Principal Component Analysis (PCA) of the environmental variables was performed to find patterns in multidimensional data by reducing the number of dimensions, with minimal loss of information. Prior to the calculation of the environmental parameter resemblance matrix based on Euclidean distance, data was checked for uniform distribution, and if necessary was log (X + 1) transformed and normalized (subtracting the mean and dividing by the standard deviation, for each variable) prior to analysis. Selective log (X + 1) transformation was required for the dissolved oxygen.

Meiofaunal assemblages

Total meiofauna density and density of individual major meiofauna taxa (individuals 10 cm^{-2}) were calculated, for each station. Non-metric multi-dimensional scaling (nMDS) ordination using the Bray–Curtis similarity measure was applied to a square-root transformed matrix of the meiofauna data in order to reduce contributions to similarity by abundant taxa, and therefore to increase the importance of the less abundant taxa in the analyses. A permutational analysis of variance (PERMANOVA) followed by pairwise comparisons was applied to test the hypothesis that significant differences in density existed between estuarine sections.

Nematode assemblages

Total nematode density (individuals 10 cm^{-2}), genus composition, trophic composition and several ecological indicators, either based on diversity: Margalef's richness Index (d) (Margalef, 1958), Shannon-Wiener diversity (H’) (Shannon & Weaver, 1963) and Pielou evenness measure (J), or on ecological strategies: Index of Trophic Diversity (ITD) (Heip et al., 1985) was calculated using the nematode dataset from each station.

The relative contribution of each genus to the average dissimilarities between sampling sections was calculated using one way-crossed similarity percentage analysis (SIMPER, cut-off percentage: 90%).

In order to investigate the trophic composition of the assemblages, nematode genera were assigned to one of the four feeding groups designated by Wieser (1953), mainly based on mouth morphology: selective (1A) and non-selective (1B) deposit feeders, epigrowth feeders (2A) and omnivores/predators (2B). Based on this feeding-type classification, the Index of Trophic Diversity (ITD) was calculated as: $ITD = \sum \theta^2$ (Heip et al., 1985) where θ is the density contribution of each trophic group to total nematode density. The reciprocal (ITD^{-1}) was used, so that the higher values of the index correspond to higher trophic diversity.

A permutational analysis of variance (PERMANOVA) was applied to test the hypothesis that significant differences in density, genera and trophic diversity existed between estuarine sections.

A DISTLM (distance-based linear model) routine was performed to analyze and model the relationship between the nematode genera assemblages and the environmental variables. The DISTLM procedure was conducted using a specified selection procedure and R^2 as a selection criterion to find the best fitting environmental variables that most suitably explains the variations in the nematode communities model (Anderson et al., 2008). The dbRDA (distance-based redundancy analysis) plot was computed to illustrate the DISTLM model.

Results

Environmental variables

The results of the environmental parameters measured at each sampling station along the estuarine are provided in Table A1 (appendix). The salinity registered progressively higher mean values from “Upstream” (26.1) to “Bay” section (34.1) following a slight decrease towards “Downstream” (32.6). The lowest salinity value was registered at sampling station 4 located at “Upstream” section of the estuary (14.5), while the highest value was registered at sampling station 35 located at “Downstream” section (35.7).

The grain size composition of the sampling stations located at “Upstream” and “Intermediate” were characterized by fine sediment, fine sand, silt and clay, “Upstream” with 79.6% and “Intermediate” with 89.6% combined mean values. The sampling stations located “Downstream” registered lower values of fine sediments with a combined mean value of 63.3%. In all sampling stations, the sediments characterized by a predominance of fine particles also presented the highest percentages of organic content (OM). The “Upstream” and “Intermediate” sections presented a mean value of 7.6 and 9% respectively. The highest OM content values were obtained in sediments of station 11 (with 12.2%) located at the “Upstream” section.

Although some variability was recorded between sampling stations, dissolved oxygen (O_2 mg/L) was similar between sampling sections, the lowest value was obtained at “Intermediate” section (7.4mg/L), while the highest values was observed at “Bay” section (7.9 mg/L). Both lowest and highest values were registered at “Intermediate” section: 1 mg/L at station 14 and 10.4 mg/L was registered at station 14A.

The temperature (°C) was also similar. At “Upstream” section was obtained the highest mean value (19.4°C) and at “Downstream section” was obtained the lowest (17.3°C). The lowest temperature values (14.5°C) were registered at stations 14 (Intermediate section) and 13 (“Downstream” section), whilst the highest values (21.0°C) were registered at stations 4 and 7 (“Upstream” section).

Registered depth values show progressively higher mean values from “Upstream” section to “Downstream” section. The lowest value (0.7m) was registered at station 10A (Upstream section) and the highest value (20m) was registered at station 37 (Downstream section). For the Principal component analysis (PCA) of the environmental variables data was untransformed, except O_2 (mg/L) which was Log (x+1) transformed, and normalized. Euclidean Distance was used as measurement for resemblance. Included data parameters in the analysis were: Depth (m), Temperature (°C), O2 (mg/L), Salinity, OM (%), Gravel (%), Coarse sand (%), Mean sand (%), Fine sand (%), Silt + Clay (%) and Manila Clam density (ind. 30^{-s}). PCA showed that the first two components (PC1, 31.9% and PC2, 19.8%) accounted for 51.7% of the variability (figure 3) of the data with the main contributors being: Silt+Clay%, OM%, Mean sand% and Coarse sand% for PC1 and Temperature(°C), Fine Sand%, O2(mg/L) and Manila clam density for PC2.

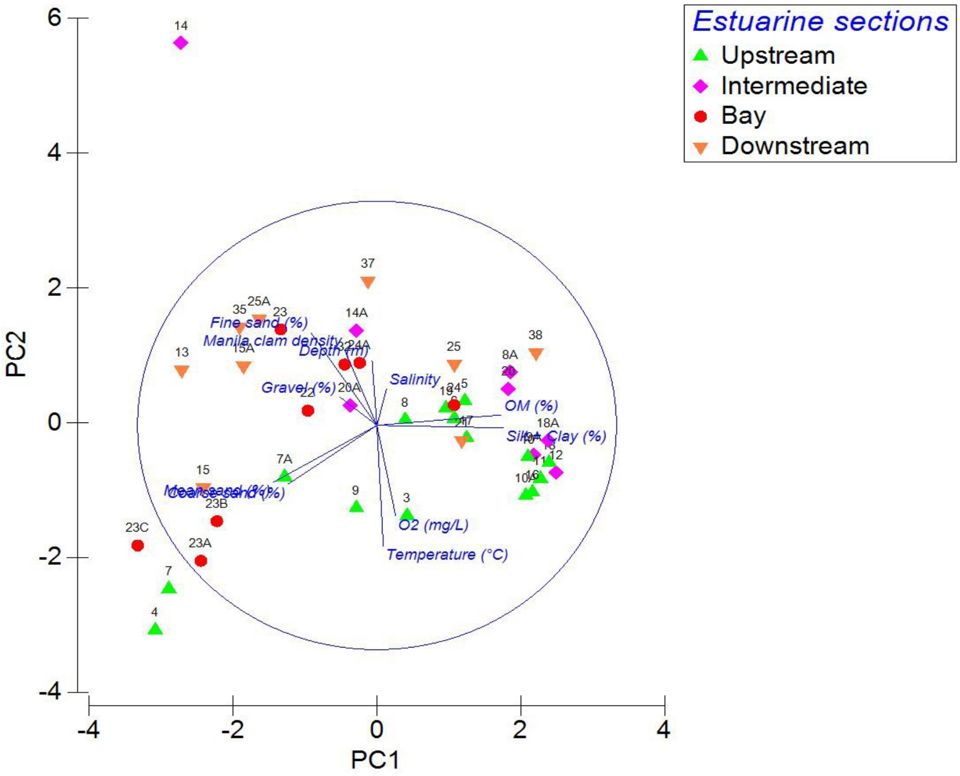

Figure 3 - Principal component analysis (PCA) ordination based on environmental data measured at each Tagus estuary section (“Upstream”, “Bridge”, “Bay” and “Downstream”).

Meiofauna assemblages – density

Total meiofauna density ranged between 11.4 ind 10 cm^{-2} ("Upstream" section, station 7) to 6829.5 ind 10 cm^{-2} ("Intermediate" section, station 20A) and the number of taxa present varied from three in the "Upstream" (station 7), "Intermediate" (station 12) and "Downstream" (station 13) sections to thirteen at "Downstream section" (station 35). The mean density of meiofaunal taxa for each section is shown in table 1.

The results of the nMDS analysis ordination applied to major taxa density of meiofauna along the Tagus estuary didn't show meiofauna spatial distribution patterns of major taxa (figure 4).

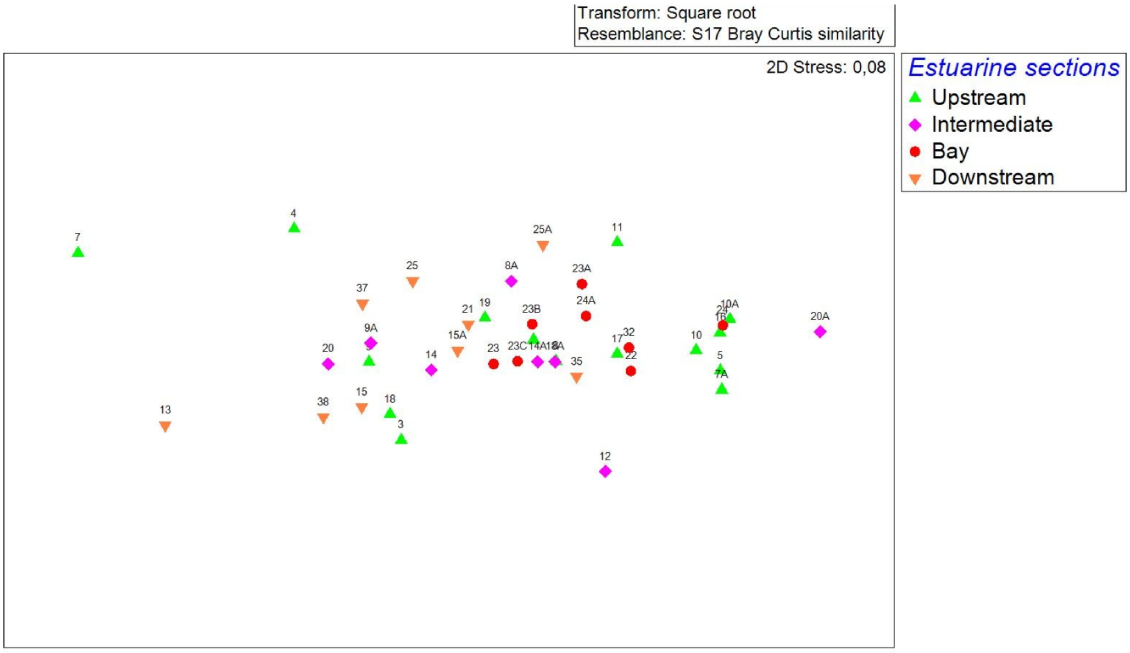

Figure 4 - Non-metric multidimensional scaling (nMDS) plot based on the meiofauna density (individuals 10cm-2) of each of the sampling stations in each Tagus estuary section ("Upstream", "Intermediate", "Bay" and "Downstream").

One-factor (section) PERMANOVA analysis of meiofauna density showed no significant differences (*p* >0.05) when all four sampling sections are considered.

Table 1 - Mean density ± SE (number of individuals per 10 cm^2) of meiofaunal taxa in each Tagus estuary section.

	Upstream	Intermediate	Bay	Downstream
Nematoda	1342,4 ± 370,7	1089,79 ± 598,0	1019,8 ± 302,8	238,3 ± 68,3
Copepoda	74,6 ± 22,8	106,42 ± 77,3	84,3 ± 22,4	18,7 ± 6,4
Polychaeta	44,6 ± 15,7	71,60 ± 57,8	44,7 ± 13,7	8,8 ± 5,1
Ostracoda	6,7 ± 5,0	19,36 ± 16,5	5,3 ± 3,0	1,2 ± 0,6
Acari	5,8 ± 4,3	3,38 ± 2,1	1,8 ± 0,9	0,5 ± 0,2
Oligochaeta	3,5 ± 1,8	1,5 ± 0,5	16,9 ± 8,6	6,2 ± 4,5
Rotifera	3,4 ± 2,7	1,43 ± 0,7	0,5 ± 13,7	0,8 ± 0,5
Cnidaria	3,1 ± 1,2	2,86 ± 0,8	2,3 ± 0,7	3,3 ± 1,1
Turbellaria	1,4 ± 0,5	0,26 ± 0,2	0,4 ± 0,4	0,6 ± 0,3
Amphipoda	0,8 ± 0,7	0,78 ± 0,6	0,6 ± 0,4	0,1 ± 0,1
Gastropoda	0,3 ± 0,1	-	0,5 ± 0,5	0,9 ± 0,3
Priapulida	0,2 ± 0,2	0,15 ± 0,1	-	0,2 ± 0,2
Bivalvia	0,2 ± 0,1	-	0,5 ± 0,3	1,5 ± 0,7
Cladocera	0,1 ± 0,1	0,15 ± 0,1	-	-
Syncarida	0,1 ± 0,1	-	-	-
Isopoda	0,1 ± 0,1	-	-	0,9 ± 0,6
Tardigrada	0,1 ± 0,1	-	0,1 ± 0,1	-
Larvae	6,1 ± 2,5	2,1 ± 1,0	8,3 ± 6,0	2,8 ± 2,0

(–) absence of taxon in the station.

Meiofauna assemblages – structural diversity

Twenty one major taxa were identified in the forty sampling stations along the estuary: Nematoda, Copepoda, Polychaeta, Ostracoda, Oligochaeta, Acari, Cnidaria, Rotifera, Turbellaria, Amphipoda, Bivalvia, Gastropoda, Isopoda, Gastrotrincha, Priapulida, Ciliophora, Cladocera, Syncarida, Tardigrada, Tanaidacea and Nauplii larvae. Nematoda presented the highest density (87.3%), followed by Copepoda (6.3%) and Polychaeta (3.7%). All other taxa attained less than 1% [*e.g*. Ostracoda (0.7%), Oligochaeta (0.6%), Acari and Cnidaria (0.3%), Rotifera (0.2%), Turbellaria and Amphipoda (0.1%) some taxa presented very low density (less than 0.05%), such as Bivalvia, Gastropoda, Isopoda, Gastrotrincha, Priapulida, Ciliophora, Cladocera, Syncarida, Tardigrada e Tanaidacea]. Nauplii larvae accounted for 0.4% of the total meiofauna density (figure 5).

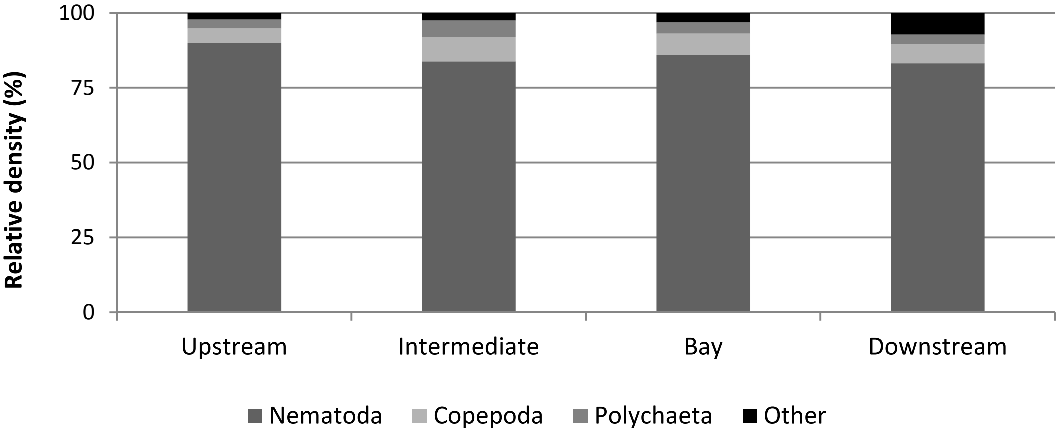

Figure 5 - Relative density (%) for the total meiofauna density at each Tagus estuary section (“Upstream”, “Intermediate”, “Bay” and “Downstream”).

Nematodes assemblages – density and structural diversity

Overall, 91 nematode genera belonging to 30 families were identified along the estuary. Most genera belonged to the orders Enoplida (25.3%), Monhysterida (22%), Chromadorida (17.6%) and Araeolaimida (13.2%) followed by Desmodorida (9.9%), and Plectida (8.8%). The orders Desmoscolecida, Rhabditida and Triplonchida were the least abundant (1.1%). The dominant families were Chromadoridae (11%), Comesomatidae, Desmodoridae and Linhomoeidae (7.7%), Oncholaimidae, Oxystominidae and Xyalidae (5.5%), Camacolaimidae, Cyatholaimidae and Sphaerolaimidae (4.4%), Axonolaimidae, Leptolaimidae, Monhysteridae and Phanodermatidae (3.3%), Diplopeltidae, Microlaimidae, Neotonchidae, Thoracostomopsidae and Tripyloididae (2.2%), Aegialoalaimidae, Anoplostomatidae, Anticomidae, Cyartonematidae, Diplogasteridae, Enchelidiidae, Enoplidae, Ironidae, Rhabdodemaniidae, Siphonolaimidae and Trefusiidae (1.1%).

Throughout the sampling stations, seventeen genera accounted for 89.1% of total nematode density: *Terschellingia*, *Sabatieria*, *Daptonema, Ptycholaimellus*, *Viscosia*, *Anoplostoma*, *Metalinhomoeus*, *Chromaspirina*, *Parodontophora*, *Chromadorella*, *Sphaerolaimus*, *Chromadora*, *Anticoma*, *Linhomoeus*, *Setosabatieria*, *Halalaimus* and *Prochromadorella*. The number of genera (S) ranged between 36 (station 35) and 2 (station 38), both located at the "Downstream" section (figure 6).

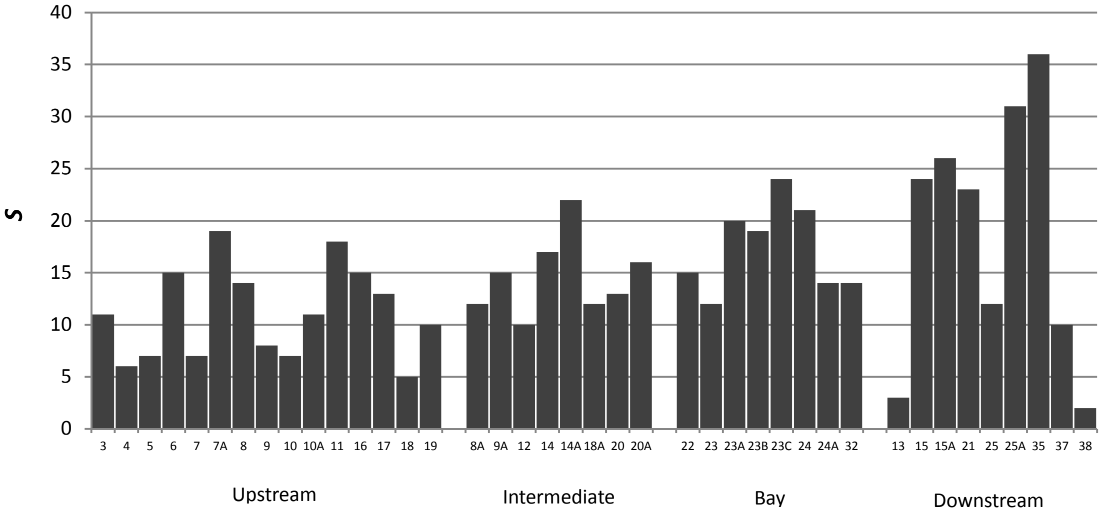

Figure 6 - Number of genera (*S*) of the nematode community in each Tagus estuary sampling station.

The three most abundant were genera *Terschellingia* (23.8%), *Sabatieria* (18.3%) and *Daptonema* (12.2%) they accounted 54.3% of total nematode density (table 2). The highest density of *Terschellingia* was observed at station 18 located in "Upstream" section of the estuary where it attained 81.7% of the total nematode density while the minimum occurred at station 12, located in the "Intermediate" section where it attained less than 1% of the total nematode density (figure 7). *Procamacolaimus* was the less abundant genus, represented 0.01% of total nematode density and it was registered only at station 37 in "Downstream" section. *Sabatieria* and *Daptonema* registered high spatial distribution pattern, they were distributed within 36 sampling stations, followed by *Terschellingia* and *Viscosia*.

Table 2- Total density (number of individuals 10 cm^{-2}), percentage and mean density ± standard error in each Tagus estuary sampling section. Only the most abundant genera (>0.5%) are included in this table.

	Total density	%	Upstream	Intermediate	Bay	Downstream
Terschellingia	9128,5	23,8	388,7 ± 140,3	251,8 ± 113,2	120,6 ± 30,2	35,4 ± 11,8
Sabatieria	7038,4	18,3	248,0 ± 92,3	195,3 ± 99,6	169,4 ± 90,5	44,6 ± 14,9
Daptonema	4692,7	12,2	208,9 ± 82,8	70,9 ± 38,2	106,0 ± 28,7	15,9 ± 5,3
Ptycholaimellus	2694,5	7,0	84,7 ± 30,4	49,7± 54,9	127,7 ± 117,9	0,6 ± 0,2
Viscosia	2315,2	6,0	50,1 ± 18,4	156,0 ± 134,6	30,3 ± 21,9	8,2 ± 2,7
Anoplastoma	1689,9	4,4	103,1 ± 48,2	15,8 ± 15,2	-	1,8 ± 0,6
Metalinhomoeus	990,5	2,6	24,7 ± 12,4	59,2 ± 68,7	10,8 ± 6,0	6,6 ± 2,2
Chromaspirina	842,5	2,2	41,41 ± 27,3	-	26,7 ± 21,3	0,9 ± 0,3
Paradontophora	794,3	2,1	14,7 ± 10,1	15,5 ± 7,6	41,8 ± 17,1	12,8 ± 4,3
Chromadorella	750,5	2,0	-	84,2 ± 108,2	9,0 ± 8,4	0,5 ± 0,2
Sphaerolaimus	518,2	1,4	29,5 ± 12,1	7,1 ± 7,9	0,5 ± 0,4	1,6 ± 0,5
Chromadora	498,1	1,3	-	33,3 ± 26,6	18,5 ± 14,0	9,3 ± 3,1
Anticoma	478,6	1,2	21,0 ± 20,3	19,0 ± 0,0	0,9 ± 0,6	0,5 ± 0,2
Linhomoeus	464,4	1,2	28,5 ± 15,4	-	0,6 ± 0,6	3,5 ± 1,2
Setosabatieria	460,8	1,2	-	49,1 ± 42,4	6,6 ± 2,1	1,7 ± 0,6
Halalaimus	460,2	1,2	22,2 ± 8,3	4,0 ± 1,8	9,3 ± 5,6	2,4 ± 0,8
Prochromadorella	368,0	1,0	0,5 ± 0,5	-	45,1 ± 42,2	-
Rhabdodemania	352,2	0,9	-	-	37,9 ± 33,5	5,5 ± 1,8
Spirinia	279,2	0,7	-	6,0 ± 8,0	6,8 ± 4,4	19,7 ± 6,6
Paracanthoncus	236,3	0,6	14,2 ± 8,6	2,3 ± 2,7	0,5 ± 0,4	0,2 ± 0,1
Neochromadora	191,9	0,5	4,2 ± 4,1	-	14,2 ± 9,1	1,7 ± 0,6
Axonolaimus	186,1	0,5	10,7 ± 8,1	-	0,5 ± 0,5	2,4 ± 0,8
Paracomesoma	177,9	0,5	-	3,1 ± 2,2	17,2 ± 7,4	1,7 ± 0,6
Paracyatholaimus	175,2	0,5	10,9 ± 10,1	-	0,5 ± 0,5	0,8 ± 0,3

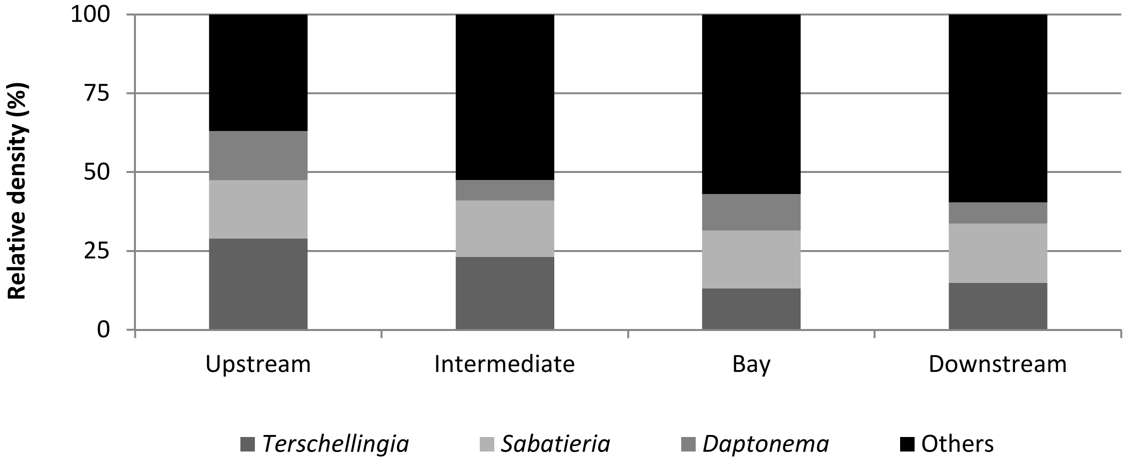

Figure 7 – Relative density (%) of the three most abundant nematode genera in each Tagus estuary section ("Upstream", "Intermediate", "Bay" and "Downstream")

The SIMPER analysis showed that stations included within the same section were heterogeneous, with a similarity within section lower than 41%. The Upstream and

Downstream section were the most heterogeneous groups (28.2% and 28.6%, respectively), while samples included in section Bays were the most similar (40.6%). Highest dissimilarities (79.1%) were observed between “Upstream” and “Downstream” sections. Differences in the abundances of *Terschellingia*, *Sabatieria* and *Daptonema* between sampling sections gave the major contributions both to similarities and dissimilarities within and between sections.

One-factor (section) PERMANOVA analysis of nematodes genera densities showed a significant (p <0.05) differences between sampling sections. Individual pairwise comparisons revealed significant differences between “Upstream” and “Downstream” (Pairwise Tests, p= 0.001), “Upstream” and “Bay” (Pairwise Tests, p= 0.002) and “Downstream” and “Bay” (Pairwise Tests, p= 0.048).

Nematode assemblages - diversity

Genera richness and structural diversity based on Margalef Index (d) and Shannon-Wiener values (H’) showed both lowest values in station 38 (d = 0,43; H’= 0,36) and both highest values in station 35 (d = 5,34; H’= 3,18) thus following the trend shown by the number of genera (Spearman correlation = 0.81; p < 0.05) in each station. Pielou evenness measure (J) showed its lowest value (0.32) at station 38 (“Downstream” section) and its highest value (0.89) at station 35 (“Downstream” section). One-factor (section) PERMANOVA analysis of nematodes genera diversity showed no significant differences (p >0.05) when all four sampling sections are considered.

Trophic composition

The index of trophic diversity (θ^{-1}) varied between the value 1.10 in “Downstream” section of the estuary at station 13 and the value 3.72 at station 35 also in “Downstream” section (figure 8).

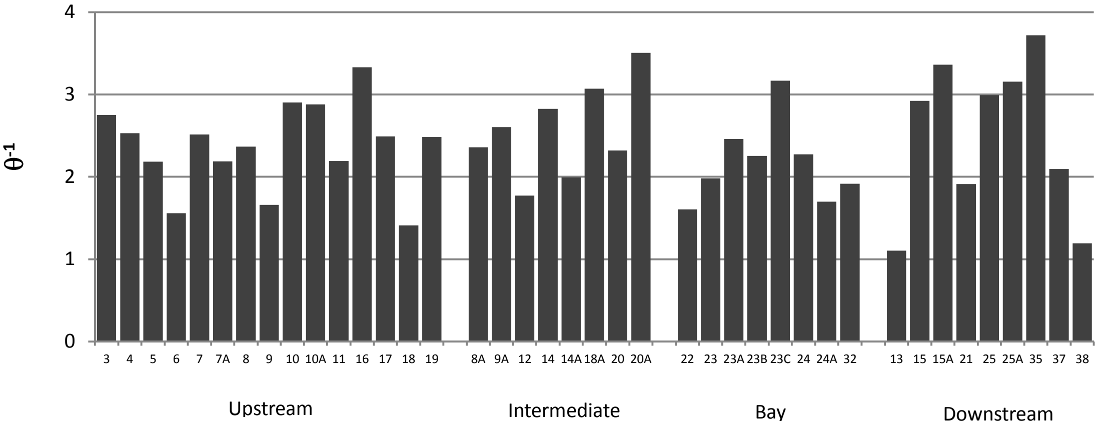

Figure 8 – Inverted index of trophic diversity (θ^{-1}) of nematode assemblages in each Tagus estuary sampling station

The trophic composition of the nematode assemblages in all sampling sections were characterized by high abundances (>40%) of the non-selective deposit feeders (1B) (“Bay” 48.4±8.6%; “Intermediate” 44.5±4.8%; “Downstream” 41.8 ±8.9% and “Upstream” 35.8±4.4%) followed by selective deposit feeders (1A) (“Intermediate” 32.9±6.5%; “Upstream” 28.3±6.6%; Bay 25.1±6.1% and “Downstream” 24±5.2%). In “Bay” sampling section, 1A feeding-type was followed by 2A (15.5±7%) and 2B (11±4.8%) feeding-types. In “Downstream” section of the estuary the third most abundant feeding group was 2B (18.2±9.2%) followed by 2A (16±4.2%) (figure 9). One-factor (section) PERMANOVA analysis on nematode feeding groups densities showed no significant difference (*p* >0.05) of the nematode trophic structure between sampling sections.

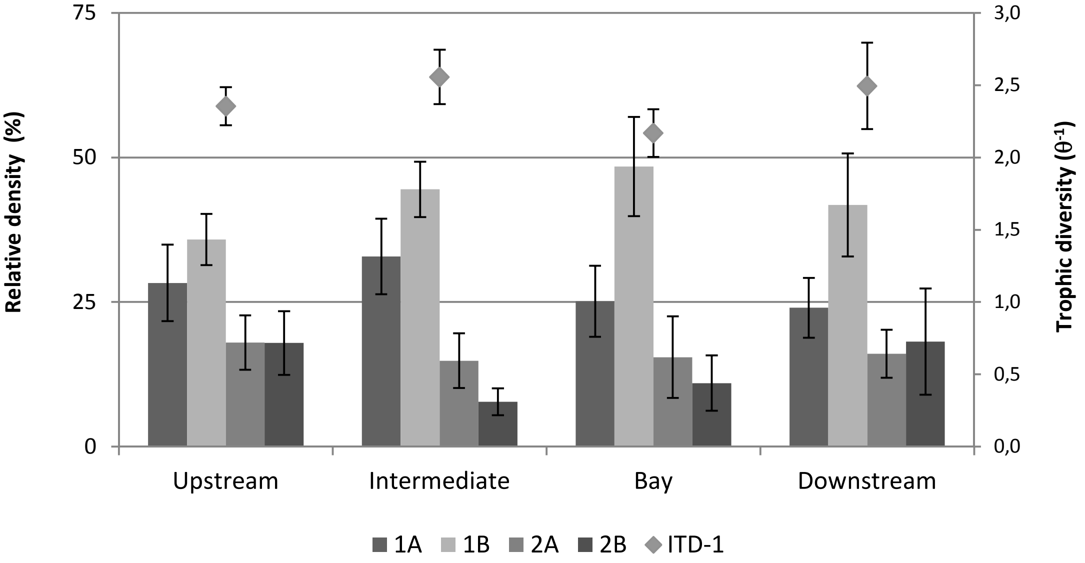

Figure 9 - Relative density (%) of the trophic groups according to Wieser (1953) and the inverted trophic diversity (θ^{-1}) for each Tagus estuary sampling section. 1A: selective deposit feeders, 1B: non-selective deposit feeders, 2A: epigrowth feeders, 2B: predators/scavengers

From DISTLM analysis, the best fitted model that explains biological patterns based on genera assemblages is represented in figure 10 and results are summarized in table A4 (appendix). The main contributors to explain the model on nematode genera variations were mean sand(%) and salinity (p = 0.001), Gravel(%) (p = 0.05) and Depth(m) (p = 0.004), describing together 28.1% of the variation.

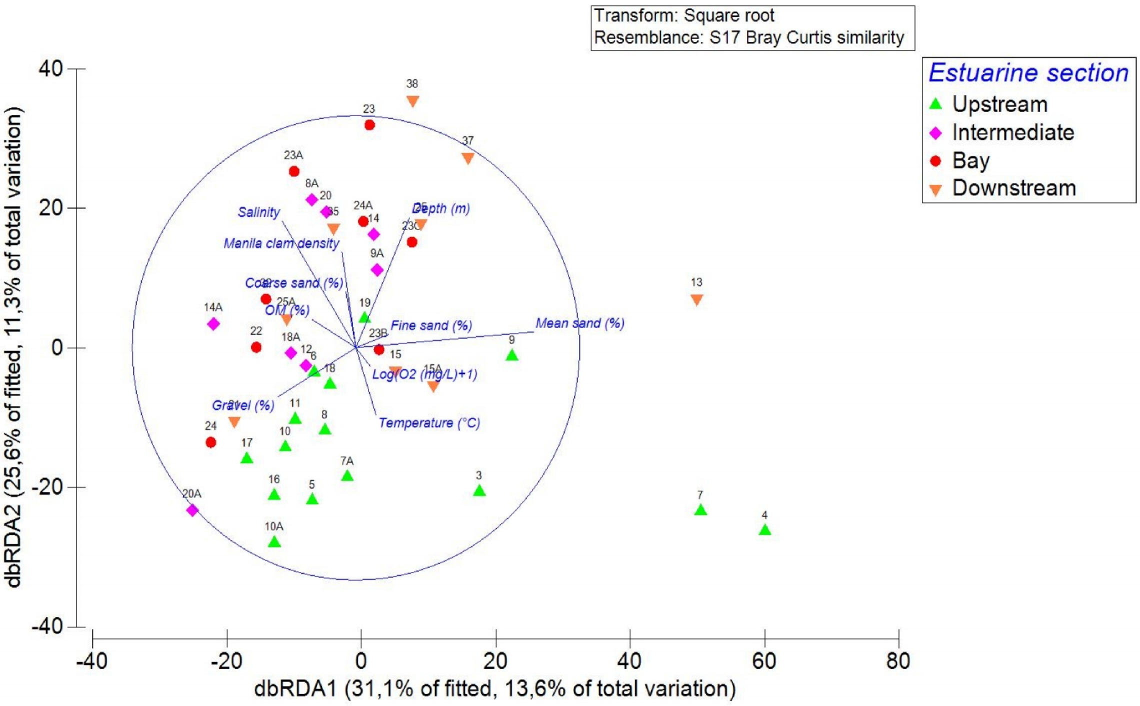

Figure 10 - Distance-based redundancy (dbRDA) plot illustrating the DISTLM model based on the nematode genera.

R. philippinarum - density and spatial distribution

In nineteen of the forty samples station no *R. philippinarum* individuals were found. In the twenty one stations where *R. philippinarum* was sampled, the densities varied between 1 (station 20A, Intermediate section) and 600 (station 23, Bay section) individuals per 30 seconds tow. *R. philippinarum* mean (±SE) densities per section are shown in figure 11.

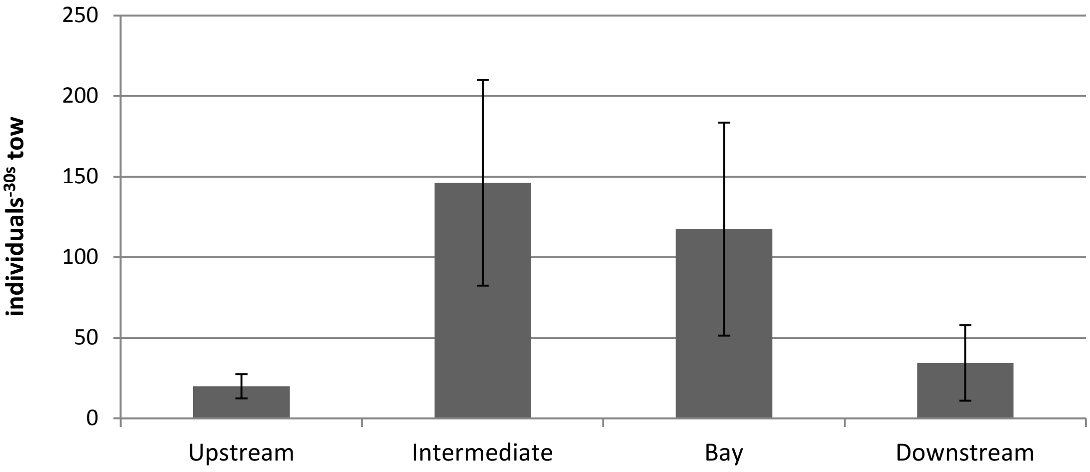

Figure 11 - Mean value (±SE) of *R. philippinarum* (individuals per 30 seconds tow) in each Tagus estuary section

Discussion

The characterization of a system based on physicochemical parameters lacks the sensitivity to determine the impact of previous events on the ecology of the system, as they only provide information about the quality at the time of the measurements (Spellman & Drinan, 2001). The biological aquatic communities, especially the sessile benthic organisms, can constitute a sort of reminiscence of the system past conditions and accurately assess ecological conditions (Caetano et al., 2013). The presence of the non-indigenous bivalve *R. philippinarum* in the Tagus estuary created the opportunity to evaluate the ability of meiofauna communities, namely nematodes, as indicators to assess disturbances in aquatic ecosystems promoted by allochthonous species.

The gradient of estuarine sediments usually shows fractions of silt and clay decreasing from the upstream towards the mouth of the estuary. This study results did not reveal that spatial estuarine pattern since the “Upstream” and “Intermediate” sections of the estuary showed higher mean percentage values of silt and clay than the lower sections “Bay” and “Downstream”. This pattern could be explained not only by the extensive mudflats of the inner estuary that are nourished by fluvial inputs of fine sediments (Freire & Andrade, 1999) but also because of the highly modified embankments and the installation of harbor infrastructures (Chainho et al., 2008) that can be promoting the fixation of the finer sediments in these areas. As expected, it also reveals a positive correlation between silt and clay sediments and higher percentages of organic content. This is explained by the high affinity of organic matter for fine-grained sediment since it adsorbs easily onto mineral surfaces and because it’s also encapsulated in clay microfabric signature (Sollins et al., 1996; Curry et al., 2007). Moreover in tide-dominated estuaries flanking environments are the main traps for fine sediments which become very rich in organic content (Dalrymple & Choi, 2007).

The higher proportions of silt and clay and concomitantly the higher organic matter content may justify the higher meiofauna density values registered in the Upstream and Intermediate sections of the estuary. The number of meiofauna taxa observed in the Tagus estuary is similar to other meiofauna communities, with densities falling within the range observed in other systems like the Mira and Mondego estuaries (Adão et al., 2009; Alves et al., 2009; 2013; Materatski et al., 2015) in Portugal. In this study, the nematodes taxon presented the highest density (>80%) in all sampling section. The abundance of nematodes over all other taxa is well documented, with Nematoda being the most abundant taxon with values normally ranging from 60 to 90% (Coull, 1999). Copepods were the second most abundant taxa followed by Polychaeta. The tendency of decreasing densities seawards obtained in this study is not according to the typical spatial patterns distribution of the nematodes densities but it follows the expected ecological patterns since nematodes density tend to increase in finer sediments (Heip et al., 1985; Boucher & Lambshead., 1995; Adão et al., 2009; Alves et al., 2009; Losi et al., 2013) and this type of sediment in Tagus estuary is located mainly on the "Upstream" and "Intermediate" sections.

The registered environmental data shows that the Tagus estuarine gradients of salinity and particles size aren't those typical of smaller estuaries with clearly defined regions. The salinity values indicate a predominantly polyhaline estuary on the "Upstream" section and predominantly euhaline on the remaining sections. These results can be explained by the marine tidal influence that extends up to 50 km inland (Guerreiro et al., 2015) and, simultaneously, by the fact that the estuary is strongly ebb-dominated due to the large extent of the tidal flats (Fortunato et al., 1999). The tide is amplified inside the estuary as a consequence of depth reduction and high current velocities and in the upper estuary the tide amplitude is reduced due to friction (Franz et al., 2014) leading to the salinity patterns registered during the sampling period.

Despite the fact the tidal regime, salinity and sediment characteristics are normally the main determinants of the meiofauna distribution in estuarine systems (Soetaert et al., 1995; Udalov et al., 2005; Adão et al., 2009; Alves et al., 2009) those patterns were not observed in the Tagus estuary. The results of this study indicated a combination of sediment type, depth and *R. philippinarum* density as major factors explaining an important portion of the total variation of meiofauna density. Nevertheless, the relationship between densities of *R. philippinarum* and meiofauna was not very clear at a lower taxa resolution as the nematode genera. Nematodes community comprised a high number of genera but it was dominated by only a few, as observed in other studies (*e.g.* Warwick, 1971; Austen & Warwick, 1989; Li & Vincx, 1993; Soetaert et al., 1995; Rzeznik-Orignac et al., 2003; Steyaert et al., 2003; Ferrero et al., 2008; Materatski et al., 2015). These communities consisted mainly on *Terschellingia*, *Sabatieria* and *Daptonema*. These genera are the most common in tidal estuarine mudflats and are highly tolerant to pollution and other distresses (Soetaert et al., 1995; Austen & Somerfield, 1997; Schratzberger et al., 2006; Steyaert et al., 2007; Gambi et al., 2009; Armenteros et al., 2009). Their high densities along the estuary may be symptomatic of a high pressure level acting over the estuarine.

Nematodes distribution pattern was mainly structured by distinct environmental factors like sediment grain size and salinity thus supporting the primary influence of the estuarine gradient on nematode community patterns (Austen & Warwick, 1989; Vincx et al., 1990; Coull, 1999; Ferrero et al., 2008; Schratzberger et al., 2008; Adão et al., 2009). Usually salinity shows greater influence over sediment grain size sometimes even overriding it. In this study it was precisely the opposite, probably because, as according to Attrill (2002) and Ferrero et al. (2008), salinity variation over time may be more important than average salinity for the distribution of nematodes along the estuary and Tagus summer and winter salinities are very similar in regular years.

In contrast to nematode density, structural diversity increased in sandy sediments. These results corroborate previous studies indicating that nematodes density tend to increase in muddy sediments, while diversity increases in sandy sediments (Heip et al., 1985; Boucher, 1990; Steyaert et al., 1999; Gheskiere et al., 2004; Muresan, 2012), probably due to the wider range of microhabitats available in sandy bottoms as compared to muddy habitats (Steyaert et al., 2003). The higher genera diversity values were registered in “Downstream” stations where environmental conditions tend to be more stable. No spatial patterns emerged based on the Trophic Diversity Index (ITD) values. ITD is generally used to correlate the trophic diversity of nematodes with pollution levels (Heip et al., 1985; Mirto et al., 2002), and statistically significant changes in this index can be obtained only when strong variations in the nematode assemblage structure occur (Moreno et al., 2008). The Tagus estuary nematode communities trophic composition was dominated by the feeding-type non-selective deposit feeders (1B) (>40%) in all sampling sections. This clearly reflects the amount and type of food present and agrees broadly with the findings of Wieser (1959) that there is an increase in the dominance of predatory (or omnivorous) species in sandy sediments and greater numbers of deposit feeders in muddy habitats. The 1B feeding-type has buccal cavity of moderate size without armature which feed less selectively because larger particles can also be ingested (Romeyn & Bouwman, 1983). Its high values suggest several hypothesis: i) an environment with high stress levels, since opportunistic genera abundances increase in adverse conditions (Gambi et al., 2003; Ingels et al., 2009), ii) the higher number of opportunistic genera may also be related to dredge fishing activity since dredging disposal leads to the proliferation of non-selective deposit feeders within the disposal sites (Schratzberger et al., 2000; 2009) and iii) opportunistic genera may benefit from high levels of deposition of organic matter as feces and pseudofeces of *R. philippinarum*. However trophic analysis conclusions based the classification of feeding complexity, as first described by Wieser (1953), should be made with care since they have the disadvantage of confining nematode species to a single trophic status which may not represent the real complexity of feeding habitats of nematodes (Moens & Vincx, 1997), with trophic plasticity being described for most feeding types (Moens et al., 2002; 2005; Schratzberger et al., 2008). The present results indicate that despite de higher abundances of feeding-type 1B, the nematode community maintained the feeding complexity with the presence of all trophic groups.

Our results corroborated previous studies (*e.g.* Gaspar, 2010) which indicated that *R. philippinarum* is currently widespread along the estuary. This situation might be due to several biological factors, such as its high tolerance to variations of environmental parameters such as salinity, temperature, dissolved oxygen, its high capability to adapt to different substratum typologies and its quick growth and sexual maturation, long reproductive cycle and high fertility (Paesanti & Pellizzato, 2000; Pellizzato & Da Ros 2005; Fernández-Reiriz et al., 2007). These features, combined with considerable genetic variability and phenotypic plasticity makes its populations, after an adaptive period that can vary between 3 and 5 years, to develop rapidly occupying a wide range of habitats (Gaspar, 2010).

A relation between *R. philippinarum* density and the pattern of the nematodes assemblages along the estuary was detected. Ólafsson et al. (2005) revealed that indirect effects of clams on the assemblage structure of meiofauna, particularly free living benthic nematodes, by hindering development of microalgal mats are much larger than any direct effect. Attraction of meiobenthic taxa and particularly nematodes to microalgal patches is often referred to in the literature (*e.g.* Blanchard, 1990; Moens et al., 2002) and has been suggested to contribute to explain patchiness and distribution of these animals in the sediment (Ullberg & Ólafsson, 2003). Once in the sediment, animals which are more sediment-bound, such as nematodes, are likely to be more affected by the presence and varying densities of the clams. Nevertheless these effects seem to be only felt when the clams densities are very high (>4.000 individuals m^{-2}) which still isn't the case of the Tagus estuary.

Conclusion

As other studies before (e.g. Coull, 1985; Vincx et al., 1990; Soetaert et al., 1995; Adão et al., 2009; Alves et al., 2009; Xuan-Quang et al., 2013), this study identified salinity and sediment grain size as two of the most important environmental characteristics determining meiobenthic communities structure and describing total meiofaunal (and particularly nematodes), density and diversity changes. But this study also revealed some evidence of the effects of *R. philippinarum* presence on meiofauna and nematode density, community structure and diversity, suggesting the need for different spatial and temporal assessments to better characterize those effects.

Benthic infauna can profoundly influence nutrients cycling through their burrow construction, bioturbation, sediment ventilation activities and feeding, (Welsh, 2003). Food is an important limiting factor for many benthic populations and since the presence of *R. philippinarum* may reduce the access of meiofauna to potential food sources or provide additional organic matter sources by the deposition of feces and pseudo-feces, the density effect shown by this study can be the result of changes in food web interactions. The identification of the trophic positions of *R. philippinarum* and meiofauna communities in the Tagus food web is essential to understand major changes related to the introduction the non-indigenous bivalve and it could be resolved by stable isotope analysis (Middelburg, 2014).

The shorter generation times of the meiofauna result in a faster potential response time to ecological incidents; and changes in the community structure take place over a time-span of months rather than years allowing early identification of disturbances caused by *R. philippinarum*. Regular meiofauna sampling could be used as a complementary tool for monitoring variation in the size and structure of *R. philippinarum* Tagus population

|GENERAL CONCLUSION

This study sought to explore potential use of meiofauna, especially free-living marine nematodes, as a biological indicator for changes in estuarine ecosystems caused by non-indigenous species. Despite the relation between *R. philippinarum* density and meiofauna distribution patterns was not directly detected, our results show that meiofauna densities and nematodes community structure in Tagus estuary are regulated not only for the environmental conditions (particularly sediment grain size and salinity) but rather by a combination of those together with biotic factors such as *R. philippinarum* density. The ecological interactions between benthic organisms (considering the possible relations established between meiofauna communities and *R. philippinarum*) implies future studies should include stable isotope analysis in order to enlighten their relation via food web since food is an important limiting factor for many benthic populations. Considering that the presence *of R. philippinarum* may reduce (or increase via deposition of feces and pseudo-feces) the access of meiofauna to potential food sources the detected density effect by our results may be due to food resource competition.

Although currently the *R. philippinarum* is one of the most important commercially exploited resources in Tagus estuary, playing a crucial socio-economic role in the subsistence of fishing communities it should also be considered as a potential threat to Tagus estuarine ecosystem. Conducting regular analysis to meiofauna communities will be critical in identifying future disturbances of the estuarine ecosystem caused whether by a sudden population increase or descrease of *R. philippinarum* or by an intensification of sediment raking by clam fishers.

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APPENDIX

Table A1 - Environmental variables measured at each Tagus estuary station

	Station	Depth (m)	Temperature (°C)	O_2 (mg/L)	Salinity	MO (%)	Gravel (%)	Coarse sand (%)	Mean sand (%)	Fine sand (%)	Silt + Clay (%)
Upstream	**3**	4,9	20,3	8,2	21,9	6,7	0,3	1,1	19,0	16,5	63,0
	4	3	21,0	8,1	14,5	0,8	0	14,1	69,6	15,5	0,8
	5	1,9	18,7	4,1	22,0	9,0	2,3	0,5	1,1	16,0	80,1
	6	3,1	18,3	7,3	32,0	8,1	0,1	0,9	7,5	16,1	75,4
	7	3,7	21,0	8,4	17,7	1,1	0,2	12,6	58,1	26,8	2,3
	7A	2,5	19,8	8,4	28,4	4,4	16,4	9,3	21,0	26,2	27,2
	8	2	19,4	8,3	30,7	7,3	2,7	0,6	2,7	37,2	56,9
	9	3,3	19,8	8,4	33,0	6,5	0,7	5,5	31,5	14,4	47,9
	10	1,2	18,5	7,4	28,1	11,1	4,0	0,6	0,8	3,5	91,0
	10A	0,7	19,0	7,7	22,8	9,5	0	0,1	0,2	1,8	97,8
	11	0,8	19,4	7,4	30,6	12,2	7,7	0,4	0,4	2,1	89,4
	16	1	19,1	8,0	25,8	9,8	0	0,2	0,2	1,8	97,8
	17	1,1	18,9	7,2	31,1	7,6	0	0,3	0,8	18,4	80,5
	18	4,1	18,9	7,4	27,8	11,4	0	0,1	0,2	1,2	98,4
	19	6,2	18,7	7,2	30,1	9,2	4,5	2,2	5,9	18,0	69,4
Intermediate	**8A**	5,8	17,9	7,1	33,0	10,7	0,1	0,3	0,7	7,5	91,4
	9A	8	19,4	8,6	32,3	11,3	0	0,6	0,8	7,3	91,3
	12	2,6	18,8	8,8	31,2	11,7	0	0,1	0,2	1,4	98,2
	14	3,6	14,5	1,0	20,4	3,2	2,3	2,6	16,1	62,3	16,7
	14A	4	17,8	10,4	31,3	7,6	28,4	1,7	1,8	25,1	43,2
	18A	2,5	18,2	7,6	31,5	11,6	0,1	0,3	0,3	2,0	97,3
	20	8,7	17,7	7,7	35,6	9,9	0,1	0,6	3,5	6,1	89,6
	20A	2	18,4	7,8	31,6	5,7					
Bay	**22**	11,5	18,7	7,9	34,9	6,0	45,3	8,7	17,7	1,8	26,4
	23	1,8	19,4	8,0	35,1	5,1	7,0	2,9	18,3	38,4	33,4
	23A	1,5	18,9	7,8	35,0	3,2	1,3	37,7	35,5	2,5	23,0
	23B	2,4	19,2	7,9	35,0	1,8	9,0	18,6	41,3	13,2	17,9
	23C	1,4	19,2	8,2	35,0	0,6	6,5	28,4	52,4	10,4	2,3
	24	2,1	17,5	7,6	30,3	10,8	26,9	3,7	2,9	2,9	63,6
	24A	9	17,2	7,7	36,5	8,1	5,9	11,1	8,5	31,0	43,4
	32	10,3	17,5	7,8	33,2	6,1	13,3	10,8	3,5	28,1	44,4
Downstream	**13**	4,5	14,5	7,3	33,3	1,3	0,1	2,0	76,7	19,9	1,2
	15	4	18,3	7,3	30,3	3,6	19,8	20,7	40,2	11,9	7,4
	15A	1,4	18,7	8,0	30,8	2,8	2,4	1,3	25,1	52,8	18,3
	21	1,4	18,3	8,1	31,3	7,9	6,7	1,5	5,9	6,0	80,0
	25	12,8	16,9	7,7	37,6	8,6	0,6	1,4	10,3	13,1	74,7
	25A	14	17,2	7,7	36,9	3,8	27,7	9,4	10,3	35,2	17,5
	35	18,9	17,4	7,7	35,7	3,2	32,5	11,2	17,2	25,7	13,5
	37	20	17,3	7,9	31,9	5,9	0	0,8	2,7	46,4	50,1
	38	15,3	17,1	7,8	35,0	11,8	0	0,4	0,7	6,9	91,9

Table A2 - Details of the one-factor PERMANOVA test and pairwise PERMANOVA comparisons tests. Bold values highlight significant effects (*p* <0.05).

	Main tests							Pairwise tests			
	Source of variation	**Degrees of freedom**	**Sum of squares**	**Mean squares**	**Pseudo-f**	**Unique perms**	***P*(perms)**		**t-value**	**Unique perms**	***p*(perms)**
Meiofauna total density	Estuarine sections	3	6294.2	2098.1	1.8819	999	0.053	**Upstream *vs* Intermediate**	0.64689	998	0.822
								Upstream *vs* Downstream	1.651	997	**0.05**
								Upstream *vs* Bay	1.094	996	0.28
								Intermediate *vs* Downstream	1.1913	982	0.162
								Intermediate *vs* Bay	1.2802	935	0.148
								Downstream *vs* Bay	2.3952	971	**0.002**
Nematode genera density	Estuarine sections	3	15483	5160.9	2.1315	999	**0.002**	**Upstream *vs* Intermediate**	1.2726	996	0.12
								Upstream *vs* Downstream	1.7568	998	**0.001**
								Upstream *vs* Bay	1.743	998	**0.002**
								Intermediate *vs* Downstream	1.1676	979	0.105
								Intermediate *vs* Bay	1.1454	927	0.157
								Downstream *vs* Bay	1.2275	978	**0.048**
Nematode trophic composition	Estuarine sections	3	4745.3	1581.8	1.434	999	0.185	**Upstream *vs* Intermediate**	0.85919	999	0.534
								Upstream *vs* Intermediate	1.4682	999	0.088
								Upstream *vs* Bay	1.0505	996	0.312
								Intermediate *vs* Downstream	1.0937	970	0.253
								Intermediate *vs* Bay	0.66043	927	0.77
								Downstream *vs* Bay	1.7641	979	**0.013**
Nematode genera diversity	Estuarine sections	3	1496.5	498.85	1.4714	999	0.183	**Upstream *vs* Intermediate**	1.5287	996	0.119
								Upstream *vs* Intermediate	1.1729	998	0.266
								Upstream *vs* Bay	2.0278	999	**0.036**
								Intermediate *vs* Downstream	0.78528	978	0.527
								Intermediate *vs* Bay	0.42523	924	0.749
								Downstream *vs* Bay	0.95525	979	0.417

Table A3 – Density (ind. 10 cm^{-2}) of meiofaunal taxa in each Tagus estuary sampling station.

	3	4	5	6	7	7A	8	8A	9	9A	10	10A	11	12	13	14	14A	15	15A	16	17	18	18A	19	20	20A	21	22	23	23A	23B	23C	24	24A	25	25A	32	35	37	38
Nematoda	202,70	37,42	3941,79	542,62	14,55	3629,94	653,85	291,06	121,62	118,50	3096,67	2944,91	457,38	1276,51	27,03	221,41	618,50	133,06	246,36	2952,18	1076,92	197,51	622,66	271,31	108,11	5461,54	239,09	1620,58	391,89	429,31	382,54	492,72	2982,33	603,95	139,29	465,70	1254,68	705,82	87,32	100,83
Copepoda	2,08	16,63	142,41	29,11		25,99	20,79	87,32	2,08	4,16	123,70	141,37	199,58		5,20	19,75	30,15	2,08	20,79	310,81	67,57	3,12	27,03	33,26	2,08	680,87	68,61	75,88	11,43	112,27	33,26	22,87	217,26	73,80	23,91	13,51	127,86	17,67	13,51	3,12
Polychaeta	4,16	3,12	40,54	11,43	1,04	244,28	45,74	8,32	5,20	2,08	30,15	90,44	84,20			3,12	4,16		7,28	56,13	48,86		50,94	4,16	2,08	502,08	2,08	9,36	13,51	133,06	42,62	7,28	68,61	44,70	2,08	17,67	38,46	48,86	1,04	
Turbellaria		4,16			1,04	1,04	2,08	1,04			2,08	2,08	1,04				1,04	1,04			7,28					0,00					3,12					2,08		2,08		
Rotifero		3,12					1,04	6,24	1,04	1,04	4,16		41,58	1,04					1,04							3,12							4,16		1,04	5,20				
Cnidaria			1,04			17,67	1,04	3,12	8,32	2,08	4,16		7,28	3,12			7,28	1,04	1,04	3,12		1,04	3,12	3,12		4,16	1,04	7,28	1,04	1,04	2,08	1,04	3,12	1,04	2,08	6,24	2,08	9,36	2,08	7,28
Ostracoda			3,12			3,12	1,04	4,16	4,16	7,28	3,12	79,00	4,16				1,04	1,04	4,16	1,04				1,04		142,41	4,16	2,08	1,04		2,08	5,20	27,03	1,04			4,16	1,04		
Gastropoda							1,04		1,04			1,04	1,04						3,12				0,01				1,04			4,16					1,04	1,04		2,08		
Cladocera								1,04					2,08										0,02																	
Oligoqueta				5,20		1,04					5,20	28,07	4,16			2,08		3,12		6,24		1,04	0,03	2,08	1,04	4,16	1,04	1,04	2,08	10,40	12,47	6,24	20,79	79,00	1,04	43,66	3,12	6,24	1,04	
Priapulida								1,04			3,12												0,04													2,08				
Acari						4,16		2,08		1,04			65,49				4,16		1,04				1,04	17,67		18,71		7,28	1,04			1,04	4,16				1,04	1,04	1,04	1,04
Amphipoda	10,40					2,08										1,04										5,20							2,08			1,04	3,12			
Syncarida																				2,08																				
Isopoda															5,20					1,04						7,28										1,04		2,08		
Bivalvia			1,04			2,08													1,04											2,08		1,04	1,04		6,24			2,08	3,12	1,04
Tardigrada												1,04																				1,04								
Ciliophora																																				3,12		2,08		
Gastrotrincha																																				7,28				
Tanaidacea																																						2,08		
Nauplii larvae				7,28				8,32		1,04		16,63	17,67				5,20			35,34	6,24			8,32	1,04	1,04	1,04	2,08		2,08			53,01	4,16	19,75	2,08	5,20		2,08	

Table A4 - Distance-based linear model (DISTLM) results for nematode genera assemblages and environmental variables**. Marginal tests**: explanation of variation for each variable taken alone**. Sequential tests**: conditional tests of individual variables in constructing the model. Each test examines whether adding the variable contributes significantly to the explained variation. Selection procedure: specified, selection criterion: R^2.

Marginal tests

Variable	**SS (trace)**	**Pseudo-F**	**P**	**Prop.**
Depth (m)	4915.7	1.9113	0.036	4,7889E-2
Temperature (℃)	5594.6	2.1905	0.009	5,4503E-2
O2 (mg/L)	1440	0.54065	0.909	1,4028E-2
Salinity	8450.8	3.4091	0.001	8,2328E-2
OM (%)	7980.8	3.2036	0.002	7,775E-2
Gravel (%)	5849.8	2.2965	0.011	5,6989E-2
Coarse sand (%)	4239	1.6369	0.056	4,1296E-2
Mean sand (%)	10823	4.4788	0.001	4,1296E-2
Fine sand (%)	3090.8	1.1797	0.266	0,10544
Silt + clay (%)	8085.9	3.2494	0.001	7,8774E-2
Manila clam density	3239.2	1.2382	0.187	3,1557E-2

Sequential tests

Variable	**R^2**	**SS (trace)**	**Pseudo-F**	**P**	**Prop.**	**Cumul.**	**Res.df.**
+Mean sand (%)	0.10544	10823	4.4788	0.001	0.10544	0.10544	38
+Salinity	0.17802	7450.4	3.2672	0.001	7,2582E-2	0.17802	37
+Gravel(%)	0.22879	5211.7	2.3701	0.005	5,0773E-2	0.22879	36
+Depth (m)	0.28088	5346.8	2.5352	0.004	5,2089E-2	0.28088	35
+Manila clam density	0.3202	4036.4	1.9668	0.02	3,9323E-2	0.3202	34
+Coarse sand (%)	0.35329	3395.8	1.6881	0.047	3,3082E-2	0.35329	33
+Temperature (℃)	0.38416	3169.1	1.6043	0.054	3,0874E-2	0.38416	32
+OM (%)	0.41269	2928.1	1.5057	0.093	2,8526E-2	0.41269	31
+O2 (mg/L)	0.42836	1609.3	0.82278	0.68	1,5678E-2	0.42836	30
+Fine sand (%)	0.439	1091.8	0.54981	0.946	1,0636E-2	0.439	29

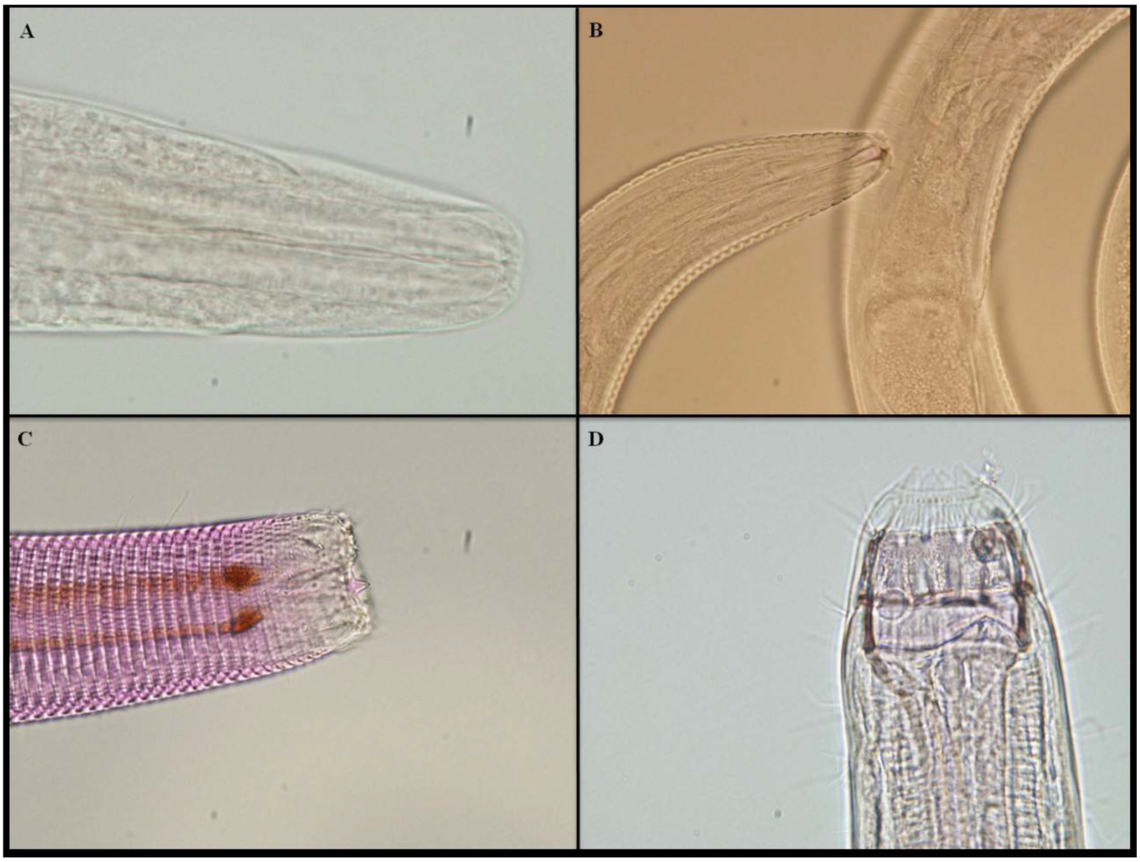

Figure A1 - Four genera of nematodes belonging to four feeding types: A - Selective deposit feeder, *Terschellingia* sp.; B- Non-selective deposit feeder, *Camacolaimus* sp.; C- Epigrowth feeder, *Euchromadora* sp.; D- Predator/omnivore, *Sphaerolaimus* sp. (Photos: P. Materatski)