UNIVERSITY OF ÉVORA

SCHOOL OF SCIENCES AND TECHNOLOGY

DEPARTMENT OF BIOLOGY

Natural recovery of the benthic nematode assemblages associated to *Zostera noltii* seagrass beds after physical disturbance caused by digging harvest activity

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Master in Conservation Biology

Dissertation

Évora, 2016

UNIVERSIDADE DE ÉVORA

ESCOLA DE CIÊNCIAS E TECNOLOGIA

DEPARTAMENTO DE BIOLOGIA

Recuperação natural das comunidades de nematodes bentónicos associados aos povoamentos de *Zostera noltii* após atividade de marisqueio

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Acknowledgements

The journey to completing a master's thesis is involved in many emotions. I doubted my capacity of putting two words together, I cried, I talked to myself countless times and I even screamed in frustration sometimes. But I mostly smiled because for each one of this states of mind I've had someone to lean on for help, advice, guidance, friendship, a kind word or simply to take a break.

I would like to take this moment to express my appreciation for each one of this individuals, and also the institutes that made this thesis come to life.

To ProMira research project (PROMAR; 31-03-02-FEP-006), MARE – UÉ (Marine and Environmental Sciences Centre – University of Évora) and Laboratory of Nematology (NemaLab) from the University of Évora.

To Professor Helena Adão and Patrick Materatski.

To Mónica Lima, to every ProMira research project collaborator, specially Professor Carlos Ribeiro, Professor Isabel Caçador, Sílvia Pedro and Ricardo Mendes, to Professor João Eduardo Rabaça, to Professor Manuel Mota and Inácia.

To Margarida Espada, Patrícia Avó and Tatiana Marques.

To my family: Ana, Mariano, Mitó, grandma Amélia and Cida.

I am very grateful for having you with me through this process. Thank you.

To the memory of my grandpa Cardoso

Contents

Natural recovery of the benthic nematode assemblages associated to *Zostera noltii* seagrass beds after physical disturbance caused by digging harvest activity

Abstract

Seagrass beds are productive ecosystems that maintain high levels of biodiversity, making them susceptible to anthropogenic pressures such as bivalve harvesting. Nematodes are considered great ecological indicators as changes in their density, diversity and structure may represent changes in the environment.

This experimental fieldwork aimed to assess the impact of the bivalve harvesting on the nematodes assemblage of a seagrass bed in the Mira estuary by simulating the digging activity. Two plots were subjected to the digging (D_1 and D_{19}) and two plots were control (C_{11} and C_{18}). The sampling took place in five occasions: T_0 – before digging; T_1 – 14 days; T_2 – 45 days; T_3 – 75 days; and T_4 – 165 days after digging.

The results showed no significant difference in the nematode assemblages' density, diversity and trophic composition between treatments and sampling times, evidencing their high tolerance for naturally stressed environments and to the level of digging they were exposed.

Recuperação natural das comunidades de nematodes bentónicos associados aos povoamentos de *Zostera noltii* após atividade de marisqueio

Resumo

As pradarias marinhas são ecossistemas produtivos que suportam elevados níveis de biodiversidade, pelo que estão sujeitos a pressões antropogénicas. Os nematodes são bons indicadores ecológicos pois respondem rapidamente a qualquer perturbação por alterações na densidade, diversidade e estrutura.

Este trabalho experimental teve como finalidade o estudo da recuperação natural das comunidades de nematodes associados aos povoamentos de *Zostera noltii* pela simulação da atividade de marisqueio. Dois plots foram sujeitos a revolvimento (D_1 e D_{19}) e dois plots serviram como controlo (C_{11} e C_{18}) e foram efetuadas amostragens em cinco ocasiões: T_0 – antes do revolvimento; T_1 – 14 dias; T_2 – 45 dias; T_3 – 75 dias; e T_4 – 165 dias após revolvimento.

Os resultados obtidos não mostraram diferenças significativas na diversidade, densidade e composição trófica das comunidades de nematodes entre tratamentos e tempos de amostragem, evidenciando a sua elevada tolerância a ambientes naturalmente dinâmicos e ao nível de revolvimento a que foram expostas.

GENERAL INTRODUCTION

Estuaries

There is no unanimous definition to what an estuary is. Through the years various classifications have been adopted (Potter et al., 2010). From its first definition given by Pritchard in 1967, that stated estuaries "*as a semi-enclosed coastal body of water that has free connection with the open sea and within which seawater is measurably diluted with freshwater derived from land drainage*", to a more recent one in which an estuary is considered transitional "bodies of surface water in the vicinity of river mouths which are partly saline in character as result of their proximity to coastal waters but which are substantially influenced by freshwater flows" (European Assemblage, 2000), estuaries have been defined differently from author to author, based on the world's region physical (*e.g.* physiography, marine based hydrographic process), chemical (*e.g.* salinity), biological (*e.g.* assemblage type) and environmental quality characteristics. Furthermore, there is a need to also include management, legal and conservation measure into its definition (Elliott & McLusky, 2002). In between, there have been a vast list of definitions that can be found in literature. Some are based on salinity level from northern and southern hemisphere and even environmental law based (Elliott & McLusky, 2002), while others prioritize physical and geomorphological classifications (Bianchi, 2007; Perillo, 1995) .

One established factor in all definitions is that an estuary has characteristics from both sea and river, even if the transition between the two of them has its own hydraulic, morphologic and biologic characteristics (Elliott & McLusky, 2002; Savenije, 2012). Estuaries show a clear salinity gradient from downstream towards upstream where saline water (euhaline 30-40 or hypersaline > 40) transition to fresh water (oligohaline 0.5-5 or freshwater <0.5). Usually a decrease in the biota diversity and richness occurs throughout the freshwater region but not in density and productivity, and is generally related with the composition and instability of the sediment (Elliott & McLusky, 2002). Also, estuaries are characterized by flowing water and sediment transport with grain size that goes to coarse to fine sediments, usually varying from less than 2 μm to over 4 mm. Simultaneously there are changes in the turbidity of the water column, nutrients, dissolved gases and trace metals (Elliott & McLusky, 2002), creating a unique type o tidal waves, a brackish environment and a funnel shape (Savenije, 2012).

These ecosystems are extremely dynamic as they are constantly facing seasonal fluctuations in freshwater input, sediment supply, wind velocity and interannual recurrence of storm events and even longer time changes in sea level and climate (Bianchi, 2007). Nonetheless, estuaries are some of the most productive ecosystems worldwide (Carvalho et al., 2013; Crooks & Turner,

1999) and encompass some of earth's highest diversity (Bianchi, 2007) from flora to fauna and avifauna, and are responsible for life support systems for humans (Lotze et al., 2014). Not only they directly provides us food in the form of plants, animals and water (e.g. industrial and agricultural use), raw materials (e.g. renewable soil) and resources (e.g. medicinal, genetic) as they are responsible in a long-term for the regulating services such as air, water, soil and climate regulation, disease control, pollination and pest control (Crooks & Turner, 1999).

In Portugal, estuaries and transitional waters are distributed along its coast, in direct contact with the Atlantic Ocean. The northern part of the country comprise semi-enclosed and shallow lagoons, characterized by a stratification of the water column inside the estuary due to high river flow that happen all year. This phenomenon leads to a tidal range of about 2 m and mean annual salinities of 20 (*e.g.* Minho; Douro). Both North-South and East-West have open coastal waters in an extension that covers more than fourteen times the transitional waters. Here the river flow is dependent on the season and the stratifications of the water column is rare and only occurs during extreme flood events such as intense rainfalls that might happen in winter months (*e.g.* Tagus; Sado; Mira) (Bettencourt et al., 2004).

Mira estuary, specifically, is located in the south-western coast of Portugal (37º40'N, 8º40'W), and together with the surrounding area forms the Natural Park of "Sudoeste Alentejano e Costa Vicentina". It is a small mesotidal system with a hydrological basin of 1576 km^2, delimited by the hydrological basins of Sado River and small Algarve rivers, in north and south, and Guadiana River and Atlantic Coast, in east and west, respectively (Loureiro et al., 1984 *in* Andrade 1986). With a semidiurnal tidal regime with amplitudes of 1 m during neap and 3 m during spring times, it forms a single channel 32 km long, and is about 400 m wide in the lower part and 40 m upstream. Its mean depths is of about 6 m (Costa et al., 1994).

Mira estuary was once considered representative of what a pristine estuary should be (Costa et al., 2001; Adão, 2004) not only for being a part of a Natural Park but because its surroundings were characterized by well-preserved eucalyptus and cork-oak woods and undergrowth. Nowadays, the estuary is still relatively undisturbed but it is possible to notice the anthropogenic presence (*e.g.* irrigated fields) that established in its surroundings through the years (Raposo de Almeida, 1996).

The physical and chemical fluctuations that influence salinity and turbidity mostly result from natural pressures due to the estuary's morphology. The region's annual rainfall distribution limits the upstream tidal penetration and degree of vertical stratification, with tidal inflow greater than freshwater outflow during the dry season. On the contrary, maximum values of freshwater can

be found during rainfall period (Andrade, 1986; Paula, 1993). The rainfall also influences the sedimentation dynamics (Paula et al., 2006).

Seagrass beds

Typically estuarine plants, seagrasses are small angiosperms that have developed physiologic, ecologic and morphologic mechanism that make them perfectly suitable to live under marine environments (Borum et al., 2004; Orth et al., 2006). Well adapted to different salinity ranges, from freshwater to hypersaline water, their growth only declines at salinities that exceed in 45 (Hemminga & Duarte, 2000). Their distribution is linked with soft substrates such as mud and sand, where they can easily expand their root system. According to their desiccation limit, some species are even able to develop in intertidal and shallow waters (e.g. *Zostera noltii*) (Hemminga & Duarte, 2000). Normally they will prefer sediments disturbed by currents or where the waves lead to patchy beds or their absence (Koch et al., 2006).

Seagrasses are composed of roots, rhizomes, leaves and flower/inflorescence, with a development through vegetative proliferation (Marbà et al., 2004). The rhizome system can extend horizontally under the sediment or vertically, pushing the leaves to the sediment surface. Their whole reproductive cycle occurs without the need of being in contact with air (Borum & Greve, 2004).

Nearly 100 million years ago, one sole lineage of monocotyledonous flowering plants has evolved in three independent lineages of seagrass: Hydrocharitaceae, Cymodaceaceae and Zosteraceae (Hemminga & Duarte, 2000; Orth et al., 2006). As most of the seagrass beds are monospecific, the species diversity is quite low. Nevertheless, seagrass beds are one of the most important coastal ecosystems, comprising high amounts of biodiversity and providing high-value ecosystem services (Orth et al., 2006). As a matter of fact, these beds are highly productive ecosystems that can hold twice as many species as the adjacent sediments (Hicks, 1986 *in* Giere, 2008). In addition to directly providing a source of nutriment for fauna, especially megaherbivores, they serve as important habitats to a large set of fauna, including important fishery species (Heip et al., 1985; Crooks & Turner, 1999; Terrados & Borum, 2004; Orth et al., 2006; Barbier et al., 2011), and provide shelter against predators and nursery for the juveniles. Anadromous and catadromous species, for instance, depend of coastal waters at some point to complete their life cycle (Heip et al., 1985).

With occurrence in every world's coastal area, the exception being the polar seas (Borum et al.,

2004; Orth et al., 2006), there are only 60 species worldwide with seagrasses being only <0,02% of the total angiospemic flora (Hemminga & Duarte, 2000; Orth et al., 2006). In Europe, the extent of seagrasses is 6,340 km^2 (Krause-Jensen et al., 2004), and there is a dominance of merely four species, *Zostera marina, Zostera noltii, Cymodocea nodosa* and *Posidonia oceanica* that are present in tropical and in temperate regions. In the north Atlantic and north pacific temperate region there is a dominance of the *Zostera marina* and temperate species of *Zostera,* respectively, while in the Mediterranean region *Posidonia, Cymodocea* and *Zostera* are the main species, with a predominance of the first one. From these European native species, *Zostera* is common in every region. Among *Zostera* species, *Zostera noltii* can be found from the southern coasts of Norway to the Canary Islands, the Mediterranean and Black Seas and south Mauritanian coast (Green & Short, 2003; Terrados & Borum, 2004). In Portugal, *Z. noltii* is present in 10 different estuaries with a distribution of about 15.74 km^2 (Cunha et al., 2013). *Z. noltii* forms thick intertidal beds. Their growth and production rate is so high that they are able to persist under stressful events (Marbà et al., 2004). This small plant is composed of 2 to 5 small narrow leaves (0.5-2 mm wide and 5 to 25 cm long) and a horizontal rhizome (0.5-2 mm thick) that is separated in various thin rhizome segments (5 to 35 mm long). Male and female flowers are present in each individual (Borum & Greve, 2004) and their shots produce hundreds of seeds (Marbà et al., 2004), with biomass variable throughout the year, reaching its peak when the temperatures and light are higher (Adão, 2004). Usually these plants colonize the intertidal zone, in the interface between marine and terrestrial environments (Moore & Short, 2006).

Primary production is one of the main features of this small plants, being responsible for 12% of the total amount of carbon stored in ocean sediments (Terrados & Borum, 2004). *Z. noltii* beds are therefore extremely efficient in fixating carbon dioxide for food webs and regulation of carbon cycle (Crooks & Turner, 1999; Hemminga & Duarte, 2000; Terrados & Borum, 2004; Orth et al., 2006). Moreover, they have a physical structuring effect, reason why they are often called ecological “engineers”. Their vast rhizomes and root network are responsible for protecting against coastal erosion and maintaining a stable shoreline (Hemminga & Duarte, 2000), not only by trapping as by storing sediments that modify waves and currents (Giere, 2008; Crooks & Turner 1999; Orth et al., 2006). Also, their leaf canopy minimize irradiance penetration, which will generate several microhabitats and retain suspended particles that will act as a filter for coastal waters and can to certain extent enhance the transparency of the water column (Giere, 2008; Terrados & Borum, 2004). Both root system and leaf canopy are able to absorb inorganic nutrients (Crooks & Turner, 1999; Orth et al., 2006), which are slowly released back into water column when the plants decompose, or are removed from the nutrient cycle by being buried in

the sediment (Romero et al., 2006 *in* Barbier et al., 2011).

Such valuable ecosystem are equally very vulnerable as they are highly exposed to human influence. Green & Short (2003) estimate losses up to 33,000 km^2 of seagrass beds over the last two decades. Lotze et al. (2006) believes that 65% of seagrasses were lost due to eutrophication, disease, destruction and overexploitation, causing a decrease in nurseries, nutrient, sediment sinks and coastal protection (Orth et al., 2006). In Mira estuary, specifically, *Z. noltii* seagrass beds disappeared almost completely in 2008, after a flood event, for reasons yet to be understood, leaving only a muddy patch of dead rhizomes (Adão, personal communication). From 2009 onwards there have been signs of natural recovery and small patches of irregular distribution (spatially and temporally) could be observed in the estuary (Cunha et al., 2013; Materatski et al., 2015; Materatski et al., 2016), although in some periods no *Z. noltii* was observed (Adão, personal communication). Such losses pose a great danger not only for the plant itself as for the faunal assemblages that live within this habitat, namely over meiobenthic assemblages, that consequently lead to the disruption of food webs and decrease species density, diversity and population biomass and disrupt all the physical component of the habitat (Grilo et al., 2012).

Some measures can be implemented in order to re-establish a seagrass population to its original condition (*e.g.* transplantation, Green & Short 2003) but it is important to be aware that this process might take decades to centuries and success is not guaranteed. Hence, preventive measures such as regular monitoring and public awareness should be taken in advance (Grilo et al., 2012).

In 2008, Mira's extensive and homogenous *Z. noltii* beds faced an almost complete loss. However, since 2009 there have been observed indications of natural recovery, with the reemergence of small patches even if in an irregular spatial and temporal distribution (Cunha et al., 2013; Materatski et al., 2015; Materatski et al., 2016).

Meiofauna

The organisms that live on – epifauna –, in – infauna – or in close proximity to the bottom substrate of marine and freshwater ecosystems are called benthos[1], vastly diverse on habitat structure and life forms. Benthic assemblages include a vast array of species from simple, single-cell organisms (*e.g.* procaryotes) to more complex animals (*e.g.* metazoans) and extremely

[1] 'Benthos' derives from the Greek word βένθος that means ´depth of the sea'

developed creatures (*e.g.* vertebrates) (Randall, 2013). The classification of benthic fauna is usually dependent on their size. Macrobenthos are organisms whose size is over 1 mm (*e.g.* bivalves and gastropods) while meiobenthos are smaller than 1 mm but are retained on a sieve with a 0.38 µm (*e.g.* nematodes and copepods) and microbenthos include organisms with a size less than 0.38 µm (*e.g.* bacteria and diatoms).

Known for many years, the term meiofauna was first used to define an assemblage of mobile or hapto-sessile benthic invertebrates (meiobenthos) distinguished from macrobenthos by their small size, in 1942, by Mare (Giere, 2008). Additionally to their size, they are also defined based on size-related life-history and feeding strategies (Warwick, 1989).

This group of metazoans is distributed from Alpine lakes to deep sea habitats, and their ecological heterogeneity is so high that several assemblages can be differentiated even within a particular habitat (Coull, 1999).

Meiofauna assemblages are conditioned by a group of abiotic factors (structure of sediment, permeability and porosity, temperature, salinity, pH and pollutants) and biotic factors (dissolved organic matter – DOM, particulate organic matter – POM, detritus, plants and bioturbation). Coull (1999), however, highlights three of them: salinity, sediment particle size and temperature.

Estuaries' particular salinity gradient will have a great influence over the meiobenthic composition and occurrence. Its surface salinity can rise up to hypersaline conditions due to high levels of evaporation and drop to freshwater during rainfall season. Nonetheless, meiofauna has a high tolerance for brackish waters, and both freshwater and marine species can co-occurs at the same environment even if marine species are in higher number than freshwater ones (Giere, 2008). Likewise, there have been observed some type of pattern in meiofauna density due to seasonal distribution, even if the recorded numbers are not equal from one year to another nor have been observed in estuaries worldwide (*e.g. Mira estuary*, Adão, 2004; *Mondego estuary*, Alves et al., 2013; Alves et al., 2015). Coull (1999) believes that this patterns can be directly related to temperature or, in a more indirect way, to controls on food density, oxygen levels bioturbation and disturbance induced by temperature.

Meiofauna is directly dependent not just from the temperature and salinity but of the grain size of the sediment that will influence spatial e structural distribution. While some investigators have observed that meiofauna reach increasing density and diversity values in finer sediments (Coull, 1999) others found that coarser and muddy sediments equally presented similar meiofauna density values, the only difference being between the taxa present in each habitat (Fonseca et al., 2011). Nonetheless, Giere (2008) estimated that in good ecological conditions

meiofauna can reach a value of $10^6 m^{-2}$.

McLachlan (1980) created an scheme for an average beach in South Africa that although not static it is possible to differentiate four strata in the sediment: 1) a dry sand stratum near the top of the shore, that only gets wet at high tide (predominance of nematodes); 2) a moist sand stratum, under the dry sand and down to the permanent water table (numerous meiofauna although dominated by crustaceans); 3) the water table stratum, where prevails low oxygen levels (moderate meiofauna with predominance of nematodes and crustacean); and 4) a low oxygen stratum, under the water table stratum (low levels of meiofauna and nematodes dominant). Above water line meiofauna can become scares due to the water content variations in tidal phase, grain size, freshwater input and high temperatures that will change de four strata profile.

This organisms will preferably live in sediments with a mean size of < 125 µm, where the species are able to move into the sediment. Moreover, most meiofauna need oxygen to metabolize. The oxic zone in muddy sediments consists of the upper 2-3 cm of sediment surface which are characterized by rich populations of a limited number of species (Tietjen, 1977; Heip et al., 1985; Moens & Vincx, 1997). When in sandy sediments, higher density of meiofauna can be found up to > 10 cm deep (Coull, 1999).

When it comes to spatial scales of meters and more, meiofauna migrates via the water column, the only difference being between poor-swimmers and deep-burrowing meiofauna to which this process occurs in a lesser degree (Palmer, 1988). Their dispersal may be influenced by taxonomic composition, hydrodynamic, underwater structures and disturbance, and both juvenile and adult meiofauna act as recruits for all types of habitat. These organisms are so diverse in their morphology and life strategies that the relative importance of the water column modes of recruitment will be different between assemblages or even within a given assemblage and its prediction will not be so easily obtained (Palmer, 1988). Water flow, on the other hand, is responsible for the passive migration of meiofauna into the water column as a result of erosion processes and advection (Palmer, 1988) and within the sediment (Boaden & Platt, 1971 *in* Palmer 1988). Meiofauna of sandy and muddy bottoms with no vegetation is present in the sediment surface and will enter the water column when flow is low, avoiding the benthic boundary layer during high tide, escaping possible passive dispersal. When inhabiting vegetated habitats, such as seagrass beds, meiofauna reach higher densities at low tide (Hicks, 1986).

Biological sediment disturbance by predators (*e.g.* macrofauna, fish) also plays a role on the meiofauna migration as it leads to sediment resuspension and consequently to an increase in

suspended meiofauna in the water column. Fish activity, specifically, is responsible for increasing to almost double copepods, foraminiferans, nematodes and the total meiofauna. This process, even though well established, need further knowledge in order to understand the frequency and extent of these disturbances and to determine if passive transport or migrations occur (Palmer 1988). When it comes to the settlement and establishment process, meiofauna can recolonize areas in days or within hours (Scheibel, 1974) and it's known that, in seagrass beds, the phytal structures may lead to higher rates of emergence of meiofaunal assemblages. This processes, however, lack many knowledge (Palmer 1988).

Although small in size, meiofauna of the estuarine sediments have a prominent ecological role (Coull, 1999). For once, they can stimulate bacterial growth that will lead subsequently to mineralization and nutrient regeneration processes. They can attract bacteria through their mucus production and are capable of breaking down mechanically detrital particles and excreting nutrients (N, C) to the habitat for bacterial use. Moreover, their bioturbation in the sediments creates a vertical conveyer within and between the sediment and the overlaying water. Gerlach (1978) also believes that meiofauna grazing on bacteria keep them in log stage and incites them to metabolize faster. Such small organisms provide, even if not directly, food for primary producers and, both indirectly and directly, for the higher levels, which lead us to their next big role. Meiofauna serve itself as a food source for higher trophic levels (Vincx, 1996). Some predators such as juvenile fish, shrimp and crabs have an obligatory meiofaunal feeding stage, primarily benthic copepods that live in muddy environments with magroalgae and seagrass beds (Gaston, 1992 *in* Giere, 2008). Nematodes, for example, have been found in the gut content of some species as well (Coull, 1990 *in* Giere, 2008). Meiofauna are able to fulfill predators' nutritional need in calories, carbon, nitrogen, proteins and micronutrients (Coull, 1999).

Additionally, meiofauna are characterized for being a stenotopic taxa which makes them important ecological flags (Austen & Widdicombe, 2006; Materatski et al., 2015). As muddy sediments inhabitants are the principal vehicle to study disturbance (natural or anthropogenic) and particularly estuarine pollution (Coull & Chandler, 1992; Coull, 1999; Schratzberger & Warwick, 1999). Their small size and rapid reproductive rate makes them reproducible and measurable over a short period of time and reasonable spatial scale. Furthermore, assemblage changes from both mortality and recruitment can be effectively accessed when the species go through a holobenthic development, which just happens with meiofauna (Coull, 1999). Amongst meiofauna, nematodes are the most tolerant of detrimental conditions such as pollution (Heip, 1980; Heip et al., 1985).

Nematodes

Nematodes represent the most abundant and predominant meiofauna taxon comprising 60-90% of the total individuals, a value only followed by harpacticoid copepods (10-40%) (Coull, 1999; Heip et al., 1985; Heip, 1980; Warwick & Price, 1979).

Assemblage wise they are smaller than macrofauna and their sampling is easier. Most marine species have between 0.2 and 3 mm, their length being 20-40 times their width (Giere, 2008). Nematodes are dioecious (separate male and female individuals), and males are usually smaller than females (Bird & Bird, 1991). Nematodes are highly diverse in respect to size, shape, number and arrangement of setae, shape of tail and amphid, buccal cavity cuticularization. Commonly, typical inhabitants of mud flats are small and have short setae and sand inhabitants are more elongated with well-sculptured cuticle, long setae and are typically carnivorous (Giere, 2008). Nematodes are also diverse when it comes to their genital organs, and their reproduction is usually by copulation (Giere, 2008). Some nematode species are ovoviviparous with the embryos developing inside eggs within the mother's body before hatching. From egg to adult stages nematodes suffer developmental changes and pass through four juvenile stages that are sometimes impossible to identify morphologically in terms of species (Warwick, 1981). Nonetheless their generation time is rapid and they lack a planktonic phase (Kennedy & Jacoby, 1999).

Estuarine nematodes seasonal spatial and temporal distribution, as the remaining meiofaunal taxa, is mostly by influenced temperature (Anderson & Coleman, 1982; Heip et al., 1985; Moens & Vincx, 2000; Fisher, 2003; Giere, 2008), salinity (Soetaert et al., 1995; Adão et al., 2009) and sediment particles size (Soetaert et al., 1995; Ndaro & Olafsson, 1999; Steyaert et al., 2003) but also bioturbation (Capstick, 1959; Heip et al., 1985), oxygen (Steyaert et al., 2007) and food availability (Moens & Vincx, 1997; Moens & Vincx, 2000; Moens et al., 2005; Vafeiadou et al., 2014), hydrodynamic regime (Steyaert et al., 2003), seagrass distribution (Fonseca et al., 2011; Materatski, 2015; Materatski et al., 2015; Materatski et al., 2016) and anthropogenic impacts (Alongi, 1985; Heip et al., 1985; Palmer, 1988; Schratzberger et al., 2002; Mirto et al., 2004; Mistri et al., 2004; Johnson et al., 2007; Lee et al., 2011; Alves et al., 2013; Alves et al., 2015).

In estuaries the salinity may vary throughout the tidal cycle, or due to heavy rainfall or evaporation, but there is a general consensus that density and diversity of marine and freshwater species, decline from the euhalyne to shallow brackish water or even freshwater zones (McIntyre, 1969; Heip et al., 1985; Soetaert et al., 1995; Coull, 1999; Giere, 2008; Adão et al., 2009; Alves et al., 2013), even if exist a predominance of marine nematodes (Capstick, 1959).

Between the depths of 2 and 5 cm, salinity usually remains constant creating a favorable refuge zone to meiofauna (Giere, 2008). In Mira estuary, Adão et al., (2009) could attest for this theory as they found low densities (39 to 229 ind. 10 cm^{-2}) and low diversity (10 to 24 genera) of nematodes from freshwater contrary to the euhaline sections that had higher densities (204 to 2234 ind. 10 cm^{-2}). Some of the predominant genera were *Paracomesoma, Odontophora, Sabatieria, Ptycholaimellus* and *Daptonema*.

Sediment particle size has a great influence over the nematode distribution, not only by controlling the presence of water in interstitial spaces as for controlling its movement that will modify oxygen and food availability (McIntyre, 1969). According McLachlan (1980) zonation previously mentioned (see Meiofauna chapter), small nematodes can be found in the sandy sediments and large nematodes are present in the water table stratum, even if there is a decrease of their density. Nematodes dominate in the below levels. In the North Sea shore the mud flats have maximum meiofauna density and richness near the low tide level while in the sandy shores the maximum values appear to be closer to mid-tidal and high-tide line (McIntyre, 1969). There is not enough information on a species level to at which extent taxa occur in the water column but *Metachromadora*, *Chromadorita*, *Ptycholaimellus* and *Prelionema* have been found there (Jensen, 1981). Gerlach (1954 *in* Giere, 2008) observed, however, that this decreasing was not as much as for other meiobenthic groups.

Usually nematodes tend to have smaller biomass (Materatski, 2015) as well as increasing densities in finer sediments (1000-5000 ind. 10 cm^{-2} in silty/fine sand), mainly up to 3 cm of intertidal muds. Their density increases as closer point to the surface is reached, with maximum values at surface 1 cm (Soetaert et al., 1995; Coull, 1999; Adão, 2004; Giere, 2008). The first upper centimeters of the mudflats hold about twice as much nematodes as in sandy bottoms up to 10 cm deep (Smith & Coull, 1987), with what seems to be a worldwide prevalence of Comesomatidae, Linhomoeidae, Xyalidae, Spirinidae and Sphaerolaimidae (Heip et al., 1985). However, it is in coarse sediments that a bigger diversity can be found (Heip et al., 1985; Tietjen, 1977), with sediments between 125 and 500 µm having a true interstitial fauna. Sediments with mean size lower than 120 µm lacking in interstitial fauna and poor burrowing fauna (Wieser, 1959). Habitats with a homogeneous sediment structure often exhibit assemblages with characteristic specialists (Vincx et al., 1990).

Nematodes can migrate in the vertical profile from 2 to 15 mm in flow conditions (Palmer & Molloy, 1986), although nematodes of intertidal sandflats respond to high currents (Fegley, 1987

in Palmer, 1988). They also respond to vibrations in the sediment surface associated with harvesting activities by moving deeper into sediments and resurfacing after (Schratzberger et al., 2004).

Sediment temperature varies throughout the day and even seasonally. Hence, nematode vertical/horizontal spatial and temporal distribution as well as development rate, generation time, egg production and hatching, movement, respiration and size will change. For instance, Anderson & Coleman (1982) observed a reduction of the nematodes generation time at high temperatures (> 30ºC) that might compromise the survival of eggs and larvae. Also, more males were produced which will lead to a decrease of the population. Nonetheless, species of nematodes that co-occur ate the same space might have different optimal temperature ranges (*i.e.* niche breadths) which will prevent possible overlapping of population development and competition is reduced. Even so, daily variation can make nematodes vertically migrate up to 20 cm deep and season variation may lead to nematode migration up to 30 cm during summer and up to 50 cm in winter (Giere, 2008).

Cycle variability due to changes in the temperature suggest that some genera could have two generation cycles. Heip et al. (1985) observed a peak of nematode density in both spring and autumn. Furthermore, in intertidal and shallow subtidal areas, the higher densities appear to be during spring and summer, a pattern that was not observed in Mira estuary (Adão, 2004; Materatski et al., 2015; Materatski et al., 2016). In the marine subtidal areas there is an identical dominance of most genera, with *Daptonema, Axonolaimus and Metadesmolaimus* juveniles representing about 50% of the assemblages through the year. In spring, however, occurs an increasing of the reproductive activity of *Paracanthonchus* and *Paracyatholaimus* (Heip et al., 1985).

Nematodes can be defined by their feeding ecology. Wieser (1953) first divided nematodes in four trophic groups according to the size of their buccal cavity structure and sediment structure: 1A – selective deposit feeders, without teeth and small buccal cavity, mostly inhabiting homogenous muds and fine sands, and sheltered algae zones; 1B – non-selective deposit feeders without teeth and large buccal cavity, inhabiting heterogeneous fine sandy sediments; 2B – epistrate/epigrowth feeders with small teeth, inhabiting sand with micro-habitats; and 2B – omnivores or predators with powerful mandibles, inhabiting coarse sandy sediments and exposed phytal (Fig. 1).

Wieser's classification was later modified by Moens & Vincx (1997) that established six trophic groups based on estuarine feeding ecology: 1) microvores (exclusively bacteria); 2) ciliate feeders (mostly ciliates and some bacteria); 3) deposit-feeders (bacteria, diatoms and other microalgae);

4) epistrate/epigrowth-feeders (diatoms and other microalgae); 5) facultative predators (detritus and other nematodes); and 6) predators (mainly nematodes). The density of each trophic group varies according to site and environmental conditions. For example, for deposit-feeders *Sabatieria* and *Theristus* the higher densities occur during autumn, winter and early spring due to the incorporation of dead *Zostera* leaves and other vegetation into the sediment (Heip et al., 1985). Nematodes are opportunistic feeders, meaning that they may change their feeding strategy as an adaptation to available food. Platt (1977) found that that there is a change in the feeding types with surface layer comprising about 30% of epigrowth-feeders, mainly Chromadoridae, with an abrupt decrease until the deeper layer when the epigrowth feeders increased again with a correspondent decrease of deposit-feeders.

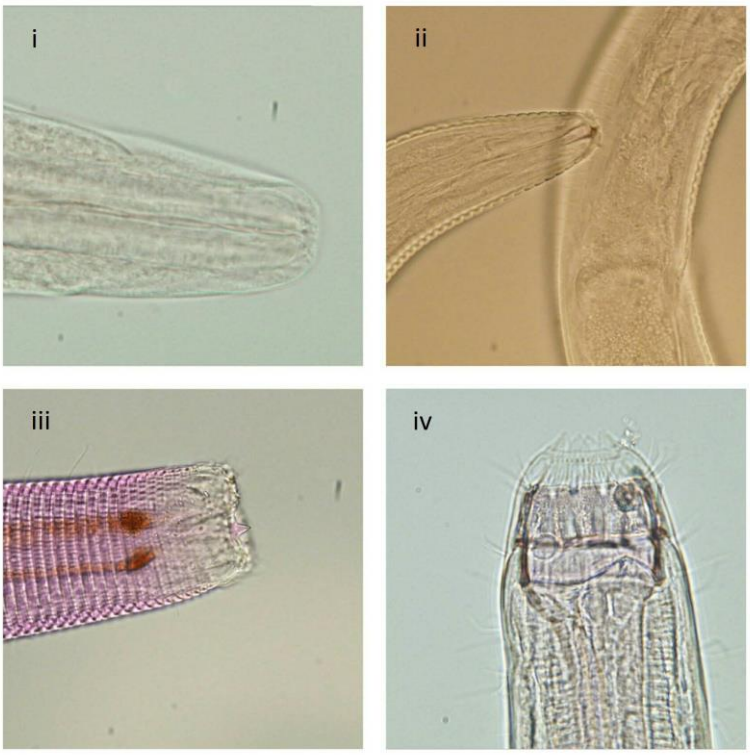

Fig. 1 Nematode genera belonging to Wieser's (1953) feeding types: i) Selective deposit feeder (*Terschellingia* sp.); ii) Non-selective deposit feeder (*Camacolaimus* sp.); iii) Epistrate feeder (*Euchromadora* sp.); and iv) Predator/Omnivor (*Sphaerolaimus* sp.) (Photos: P. Materatski)

Nematodes high occurrence rates in the benthos alongside their central role in the trophic webs of aquatic ecosystems (Coull, 1990; Coull, 1999) and capacity of stabilizing the effects of shores makes them of exceptionally ecological importance (Platt & Warwick, 1980). Changes in their density, diversity relate to different types and levels of stress. Assemblage structure and

functioning might also relate to alterations in the ecosystem (Kennedy & Jacoby, 1999). The condition of their assemblages can be analyzed based on multiple parameters, namely density, diversity (*e.g.* Shannon-Wiener diversity, *H'* and Margalef Index, *d*), body size, biological trait analysis, life strategies (*e.g.* Index of Trophic Diversity, *ITD* and Maturity Index*, MI*) and trophic guilds. The Index of Trophic Diversity (*ITD*) and the Maturity Index (*MI*) are two of the most used ecological indices to study nematodes' assemblage response to changes in environment, whether natural or anthropogenic. The first is based on the Wieser (1953) feeding type classification and usually links trophic diversity with environment pollution (Heip et al., 1985) and the latter is based on the nematodes' life strategies in when under disturbance situation (Bongers, 1990; Bongers et al.,1991). *ITD* ranges from 0.25 (highest trophic diversity) and 1.0 (lowest diversity) and usually is used its reciprocal value (θ^{-1}) so that the highest values correspond to the highest trophic diversity. *MI* assign a *c-p value* to each nematode genera that associated with r- and K- life-history strategies, that ranges from 1 (colonizers) to 5 (persisters). Colonizers are typically taxa with rapid growth and reproduction and high tolerance to disturbance while persisters are more sensitive taxa, with slow growth, that can survive in fairly stable and pristine environments (Bongers, 1990; Bongers et al., 1991). In general, changes in both indices are only highlighted when the variations in the nematode's assemblage structure are remarkable. For this reason, neither of them should be single used in monitoring programs (Moreno et al., 2011; Vincx & Heip, 1987).

Anthropogenic pressures

Pickett & White (1985) defined disturbance as 'any discrete event in time that disrupts ecosystem, assemblage, or population structure and changes resources, substratum availability, or the physical environment'. The human influence in estuaries is not recent with records of its presence with ten thousand years. People settle near water firstly attracted by the food it would supply, then for commerce purposes, as aquaculture started to be viewed as a mechanism that could reply to the high demand for food (Costa-Pierce, 2002 *in* Dumbauld et al., 2009).

Even though anthropogenic pressures may simulate natural disturbances, its extent nowadays is bigger than the ecosystem can support (Paine et al., 1998 *in* Dumbauld et al., 2009). Depending on its frequency and intensity, such activities may have an impact on the structure and functioning of the ecosystem (primary production, nutrient cycling and assemblage structure) (Day et al., 2013).

Day et al. (2013) identifies four categories of an ecosystem's disturbance: enrichment through

high levels of organic material (*e.g*. eutrophication, specie's secondary metabolites), inorganic matter (*e.g.* urban sewage, industrial effluents) or thermal additions, physical alterations (*e.g*. oil exploration, dredging, navigation), introduction of toxic materials and assemblage disruption through introduction of exotic species or harvesting. The latter is an activity with great social, economic and cultural importance (Oliveira et al., 2013).

Portugal has a long tradition of producing and harvesting bivalves. In fact, it has a consumption *per capita* of 58.5 kg/person/year, one of the highest of the world. Its production requires almost no husbandry and techniques used are very simple. Besides, there are no costs implied to their production (Oliveira et al., 2013). The legislation for harvesting activities in Portugal only allow hand gathering or rudimentary fishing tools used by licensed persons but in reality the number of non-authorized person harvesting bivalves is immense (Cunha et al., 2005).

The digging activity involves the physical disturbance of the estuary as it consist in the turnover of the sediment, and bivalve collecting not only directly affects the dynamics of the target species (McLusky et al., 1983) as the associate fauna that also inhabits the subtidal and intertidal sediment. Organisms get exposed to predation and desiccation or, on the contrary, get buried and biogenic structures that contribute to the oxygenation and stabilization of sediments are removed (Brown & Wilson, 1997). Depending on the disturbance intensity and frequency it can lead to the displacement of species to adjacent unfavorable habitats or, in extreme cases, complete defaunation of disturbed areas through direct mortality and physical damage (Hall, 1994). Sediment texture and composition and chemical environment get disrupted and smothering of adjacent habitat can occur due to the resuspension resuspension of particles of the upper layers of the sediment and to the displacement of this layer that can be carried away by tidal current that lead to subsequent deposition of fine sediments (McCall, 1977). In some cases, the sediment surface might be removed (*i.e.* aggregate extraction) and the remaining sediment will be completely different from the previous one. In this case, the environment might be unsuitable for recolonization by the species that usually lived there (Kenny & Rees, 1996).

Nonetheless, Grime (1973) introduce the Intermediate Disturbance Hypothesis (IDH) that states that disturbance events are important in maintaining species diversity by preventing the competitive exclusion by dominant species within an assemblage. This theory works on the following assumptions: a assemblage subject to very high levels of disturbance will have an increase in diversity as soon as the disturbance level decreases, whilst a assemblage with low rates of disturbance will exhibit an increase in diversity when disturbance increases sufficiently to prevent competitive exclusion of certain taxa by dominant species. Thus, physical disturbance controls the spatial and temporal heterogeneity of marine soft sediment assemblage within a

habitat. Likewise, other investigators support that sediment disturbance and benthic species density and diversity don't need to be necessarily negatively correlated as far as the sediment surface is not removed from its original place or its composition is not affected by the sediment turnover, no change occurs in the seagrass beds vertical profile, which attenuate potential effects of the digging (Webb & Parsons, 1991; Carvalho et al., 2013) or will be less affected in long-term (Hall, 1994 *in* Giere, 2008). In addition, the turnover of the sediment can bring to surface buried organic matter and increase food availability for deposit feeders that could also promote benthic resilience (Miller et al., 1984; Schratzberger & Jennings, 2002).

Dernie et al. (2003) suggests that recovery rates following physical disturbance event could be linked to the sedimentological characteristics of the habitat. In general, assemblages of nematodes that are usually exposed to dynamic ecosystems as estuaries can recover quickly after disruptive events as their species are adapted to high levels of natural disturbance as inhabitants of naturally stressed environments (Collie et al., 2000). Benthic fauna inhabiting subtidal muddy sand sediments are more tolerant and resilient to disturbance than those from subtidal mudflats (Schratzberger & Warwick, 1999; Collie et al., 2000; Dernie et al., 2003), as the natural background disturbance regime in sandy bottoms is more energetic (Collie et al., 2000). However, depending on the type and level of disturbance, it is possible to observe a reasonably rapid recovery rate of benthic infauna of the estuarine mudflats (Kaiser et al., 2001; Skilleter et al., 2005), not only for macrofauna (*e.g.* Brown & Wilson, 1997; Dernie et al., 2003; Morello et al., 2005; Carvalho et al., 2013) but also for meiofauna (*e.g.* Sherman & Coull, 1980; Pranovi et al., 2003; Dye, 2006), namely nematode assemblages exposed to various natural (*e.g.* tidal cycle) and physical disturbances (*e.g.* bivalve harvesting, bait-digging, crab-tilling) (Alongi, 1985; Schratzberger et al., 2002; Mirto et al., 2004; Mistri et al., 2004; McLaughlin et al., 2007; Lee et al., 2011). In general, small-bodied, motile and opportunistic benthic species are capable of recovery more rapidly, from hours to days (Sherman & Coull, 1980; Warwick, 1986; Pranovi et al., 2003; Mistri et al., 2004; Johnson et al., 2007) or months (Dayton et al., 1995; Kaiser et al., 2001; Dernie et al., 2003; Mirto et al., 2004; Dye 2006; Lee et al., 2011), whilst large-bodied and relatively sessile species and assemblages that contain biota responsible for the stability of sediments and biogenic structures are less tolerant of physical disturbance. In this case, the recovery might take years instead of days or months (Dayton et al., 1995; Collie et al., 2000).

Sediment composition plays a determinant role in the assemblage's recovery but is not the only factor to influence this process. Hydrodynamics, particle size and a variety of environmental and chemical factors can be determinant for the recovery depending of the site (Dernie et al., 2003). Likewise, the season at which the disturbance occurs can also have some influence over the

recovery process, as the recruitment supply of larvae and adult infauna will be different (Dernie et al., 2003).

The benthic assemblages recovery related to disturbances not only from bivalve harvesting but to bait-digging, hand collection of cockles, hydraulic dredging and fish trawling are likely to occur via active migration or passive transport from undisturbed adjacent areas (Palmer, 1988) and through the migration of adults (Dernie et al., 2003), and also due to meiofauna high intrinsic growth potential and highly plastic reproduction strategies (Alongi, 1985).

Anthropogenic disturbed estuarine environments usually have benthic infaunal assemblages composed of small, r-strategist organisms, with high density of few species and low diversity and low individual biomass with capacity to produce high biomasses. Moreover, the organic matter content (*e.g.* sewage) that is accumulated by the fine sediments creates a suitable environment for detritus and deposit feeders in areas that present low hydrodynamic energy, where the food is the main limiting factor rather than space. These assemblages have an inherent ecological tolerance of environmental variability, as the estuaries can absorb stress more effectively than other ecosystems. Their tolerance for detrimental condition, namely low oxygen and low and/or variable salinity is very high and thus they can recover from disturbance at the individual, population and assemblage level (Elliott & Quintino, 2007; Elliott & Whitfield, 2011).

The majority of studies presented a fairly quick recovery of the meiobenthic assemblages, but (McLaughlin et al., 2007) found a biomass decreasing of a *Zostera* seagrasss bed after three months of hand-racking activity. This information suggests that while harvesting can be considered a relatively low impact activity, *Zostera* beds must be avoided, or at least monitored in order to regulate the intensity to which the activity occurs and to limit the number of harvesters and techniques used.

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Natural recovery of the benthic nematode assemblages associated to *Zostera noltii* seagrass beds after physical disturbance caused by digging activity

Keywords

Benthic nematode assemblages / Bivalve harvesting / Seagrass beds / Natural recovery / Experimental fieldwork

Abstract

Highly productive ecosystems of *Zostera noltii* seagrass beds are especially susceptible to the digging activity that results from the bivalve harvesting. The digging of the sediment can lead to changes of its biochemical composition which will affect the plants spatial distribution and might affect its associated benthic assemblages' dynamics. Benthic nematodes are considered good ecological indicators of aquatic habitats as changes in their density, diversity, structure and behavior may represent changes in the environment. This experimental fieldwork aimed to assess the impact of the digging activity on the benthic nematode assemblages associated to sediments of the *Z. noltii* seagrass beds of Mira estuary (SW Portugal), by evaluating their diversity, density and genera composition. The following null hypothesis H_0 was tested: There are no significant differences in the density, diversity and trophic composition of nematodes assemblages during the natural recovery of the nematodes assemblages. Hence, two plots were subjected to the digging (D_1 and D_{19}) and two plots were control (C_{11} and C_{18}) and the sampling took place in five different occasions. The nematode assemblages were correlated with environmental parameters determinant for their spatial and temporal distribution patterns: water content, salinity, sediment nutrient composition and organic matter content. The results revealed no significantly differences for the environmental parameters nor for the nematode assemblages' diversity, density and trophic composition between treatments and sampling times, evidencing their high tolerance not only for environments naturally stressed as for the level of digging they were exposed. Nonetheless, further research is needed to comprehend the extent of their resilience, namely by increasing the intensity and frequency of the digging events.

Introduction

Seagrass beds are one of the most productive ecosystems comprising high amounts of biodiversity, equally in density and diversity (Orth et al., 2006). With worldwide occurrence, they are usually considered "ecosystem engineers" as their vast rhizome and canopy system reduce the hydrodynamic energy from currents and waves, stabilize sediments and protect the coastal shores from erosion (Crooks & Turner 1999; Hemminga & Duarte 2000; Orth et al., 2006; Giere, 2008). Moreover, they provide habitat and a source of nutrients for fauna (Heip et al., 1985; Crooks & Turner, 1999; Terrados & Borum, 2004; Orth et al., 2006), serve as shelter from predators and nursery for the juveniles (Orth et al., 2006).

Amongst all fauna that inhabits seagrass beds, there is one with an important ecological role – meiofauna, methodologically defined as capable of passing through a sieve of about 1 mm but getting retained in a sieve with mesh 0.38 µm (Warwick 1989). These animals are present in every marine sediment and Giere (2008) has estimated that their density could reach $10^6 m^{-2}$ in favorable ecological condition. Its spatial and temporal distribution patterns in estuaries are mainly driven by estuarine gradients of salinity, grain size and temperature (Coull, 1999). Vertically, meiofauna shows higher densitys in the surface decreasing with depth (Soetaert et al., 1995; Coull, 1999; Adão 2004; Giere, 2008; Alves et al., 2015; Materatski et al., 2015; Materatski et al., 2016). They will preferably inhabit particles with mean size < 125 µm, where the can reach twice as much as in sediment with higher mean size particles (Smith & Coull, 1987). It is a known fact that this organisms will have a decline in its distribution and density as one goes from euhalyne towards freshwater (Austen & Warwick, 1989; Adão et al., 2009; Alves et al., 2013).

Nematodes are the most abundant meiofaunal taxon, comprising about 60 to 90% of the total meiofauna (Heip et al., 1985; Coull, 1999). This particular group of metazoans have a prominent ecological role (Coull, 1999). Not only they represent a food source for the higher levels as they can simulate and promote bacterial growth that are important to mineralization and nutrient regeneration processes which directly or indirectly provide another food source for the higher levels (Gerlach 1978; Vincx 1996). As muddy sediments inhabitants, nematode are considered good ecological flags (Austen & Widdicombe 2006), and are vehicles to assess natural and anthropogenic disturbance, particularly estuarine pollution (Coull & Chandler 1992; Coull, 1999; Schratzberger & Warwick 1999). Their small size, rapid reproductive rate and absence of planktonic phase makes them reproducible and measurable over a short period of time and reasonable spatial scale (Coull, 1999).

Such rich habitats as *Z. noltii* seagrass beds are very prone to anthropogenic disturbances,

namely through the bivalve harvesting, a very common activity in European estuarine ecosystem (Carvalho et al., 2013). In Portugal, particularly, bivalve harvesting has a long tradition, with an estimated consumption rate *per capita* of 58.5 kg/person/year (Oliveira et al., 2013). Activities that cause physical disturbance of sediments not only affect the biological assemblages that inhabit those habitats (McLusky et al., 1983) by exposing species to desiccation and predators or burying them, with consequent removal of biogenic structures that are important for the oxygenation and stabilization of the sediments (Brown & Wilson 1997). Bivalve harvesting can also lead to the benthic infauna to migrate to adjacent habitats less suitable for them and, depending on the intensity and frequency of disturbance, to the complete defaunation due to physical damage or direct mortality (Hall, 1994).

Nematode assemblages are usually very tolerant and resilient under pressure. Some investigators consider that sediment turnover and species density and diversity could not be negatively correlated as long as the sediment composition is not affected during the turnover, no change occurs in the seagrass beds vertical profile and, consequently, the digging effect will be less pronounced (Webb & Parsons, 1991; Carvalho et al., 2013) or, at least, will be affected in long-term (Hall, 1994 in Giere, 2008). Moreover, it has been observed a quick recovery, from hours to months, of the nematode assemblages of the intertidal mudflats of estuaries as they are highly exposed to natural perturbation such as tidal forces and currents that are a structuring characteristic of this habitat (Alongi 1985; Schratzberger et al., 2002; Mirto et al., 2004; Mistri et al., 2004; Johnson et al., 2007; Lee et al., 2011).

At Mira estuary, the extensive *Z. noltii* beds faced an almost complete loss in 2008 with the declining causes still unknown, in spite the many studies developed (Cunha et al, 2013; Materatski et al., 2015; Materatski et al., 2016 and research project ProMira). From 2009 onwards there have been observed a natural recovery of the beds (Materatski et al., 2015; Materatski et al., 2016). This study provides the opportunity to understand if the Mira's bivalve harvesting activities could have stimulated the habitat loss.

This main aim of this study was to assess the impact of the bivalves digging on the benthic nematode assemblages of the seagrass beds of the Mira estuary, through an experimental fieldwork. The spatial and temporal distribution of the nematode assemblages was investigated before and after the digging activity and during the natural recovery of the seagrass bed habitat, and was connected with important environmental variables for the nematode assemblages. The following null hypothesis H_0 was tested: There are no significant differences in the density, diversity and trophic composition of nematodes assemblages during the natural recovery of the nematodes assemblages.

Material and Methods

Study area

Sampling was performed at the Mira estuary, at the south-western coast of Portugal (37º40'N, 8º 40'W), which is included in the Natural Park of "Sudoeste Alentejano e Costa Vicentina" and therefore considered a relatively undisturbed estuary (Costa et al., 2001). Mira estuary is a small mesotidal system with a semidiurnal tidal regime with amplitudes of 1 m during neap and 3 m during spring times. It is formed by a single channel and is usually up to 400 m wide that reaches a maximum depth of 6 m (Costa el., 1994). These characteristics leads to a tidal influence up to ca. 40 km upstream. The lower section of the estuary has a dominant marine influence due to the seasonal low and limited freshwater input (Paula et al., 2006).

The physical and chemical fluctuations mostly results from natural pressures due to the (i) estuary's morphology as its terminal section is rather regular and facilitates the upstream tidal penetration; (ii) the dynamic sedimentation that promotes an accumulation of coarse sediments near freshwater and sea and muddy sediments in the lower and medium sections of the estuary; (iii) a normally reduced outflow determined by the Santa Clara dam during the 1960s and the region's annual rainfall distribution (from January to March) limits the upstream tidal penetration, with the rest of the year being usually dry (Paula et al., 2006).

The Mira estuary was characterized by an extensive and homogenous *Z. noltii* and *Z. marina* beds in the adjacent subtidal area until 2008 when it faced an almost complete loss. To this day, the causes for this collapse are still unknown, even though various studies have been developed since 2010 (Cunha et al, 2013; Materatski et al., 2015; Materatski et al., 2016 and research project ProMira). Nonetheless, since 2009 the *Z. noltii* bed has been showing signs of natural recovery, characterized by growth pulses with an irregular spatial and temporal distribution of small-size patches (Materatski et al., 2015; Materatski et al., 2016).

The experimental setup was developed in the intertidal seagrass bed of Z. noltii (37º43'N, 8º45'W), located near a private rustic style accommodation where the seagrass meadows are more protected from the harvesting activity as the access to this area is restricted (Fig. 2). Moreover, a previous study developed during February 2015 to evaluate the ecological condition of the seagrass beds under natural recovery, showed the good environmental condition of the area selected for the experimental fieldwork.

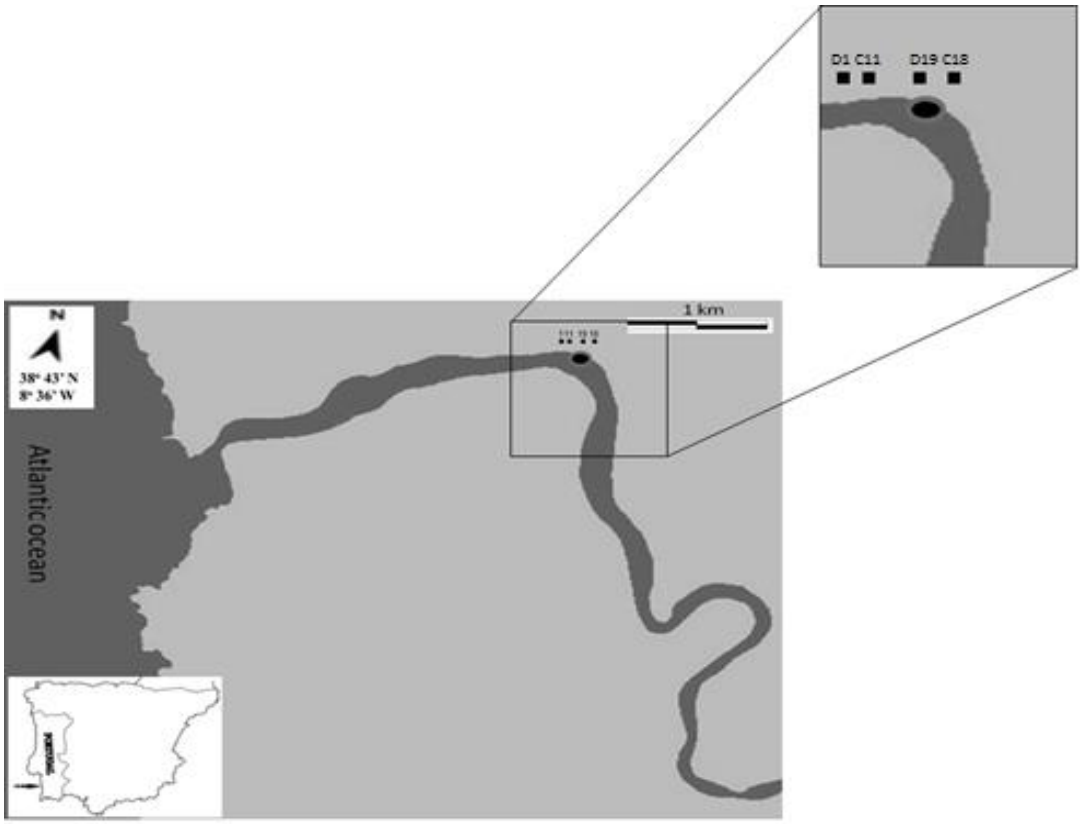

Fig. 2 Mira estuary (Portugal): indication of sampling site (*black circle*) and detailed localization of control (C_{11}, C_{18}) and digging (D_1, D_{19}) plots.

Experimental design

In order to evaluate the effect of the digging activity over the nematode assemblage, the selected area was divided by plots with 4 m width and 20 m length, and a total of four experimental plots were randomly demarcated through spatial analysis methods. Each plots was established *in situ* and was divided in 16 subplots, distanced 1 m to each, with a buffer area of 10 cm inside them, and 2 m between subplot rows to preserve the sublplots during the sampling procedure.

Once this experiment aims to simulate the digging activity, two plots were subjected to the disturbance ("Digging" – plot D_1 and plot D_{19}) and the remaining two were control plots ("Control" – plot C_{11} and plot C_{18}).

The sampling took place during low tide, in five different sampling occasions: T_0 – before digging; T_1 – 14 days after digging; T_2 – 45 days after digging; T_3 – 75 days after digging; and T_4 – 165 days after digging. At each sampling occasion, 3 sublplots were randomly selected and sampled for biological data, *Z. noltii* seagrass, grain size analysis (3 replicates), organic matter, nutrient and

interstitial pore water salinity analysis (5 replicates). No subplot was sampled twice. The results presented in this study include the T_0, T_1 and T_3, considered representative of the nematode response to the physical disturbance of the sediment by the data analysis.

Sampling and samples treatment

Environmental data

Salinity, temperature (ºC), pH and Eh (c) of the overlaying water above the sediment were measured *in situ* using a VWR pHenomenal MU600H with pHenomenal 111 electrode and pHenomenal OXY 11 probe. Additionally, at each site and sampling occasion, five sediment samples were collected by forcing a hand core (5 cm inner diameter) to a depth of 10 cm and collected in a decontaminated container and frozen until further laboratorial analysis of N and P nutrients (µmol L^{-1}). Ammonium (NH_4 - N) determination was based on the formation of the Indophenol Blue (Koroleff, 1983 *in* Grasshoff & Johannsen, 1972) and nitrate (NO_3^- - N), nitrite (NO_2^- - N) and phosphate (PO_4^{3-} - P) concentration were determined by an adaptation to Koroleff's protocol (Koroleff, 2007). Relative humidity of the sediment was calculated measuring the fresh weight of the sediment and its dry weight after dried in oven at 60º C until its complete stabilization. Total organic matter was measured following the *Loss in Ignition* (LOI) method (Heiri et al., 2001). Also, three sediment samples were collected by forcing a hand core (5 cm inner diameter) to a depth of 10 cm and collected in a 100 ml decontaminated container and were frozen until further laboratorial analysis. All samples were analyzed using a Coulter Laser Light Scatter 230. The following size categories of sediment were determined: mean grain size, clay (< 0.004 mm), silt (between 0.004 and 0.063 mm), sand (between 0.063 and 2 mm) and gravel (> 2 mm). The relative content of the different grain size fractions was expressed as a percentage of the total sample weight. The photosynthetic efficiency (α) of *Z. noltii* beds was measured *in situ* using a pulse-amplitude modulation (PAM) fluorimeter in light-adapted plants and in plants kept in the dark for a 15 minutes period.

Biological data

Nematode samples were collected by forcing a hand core (3.6 cm inner diameter) to a depth of 3 cm. The respective samples were preserved in a 4% buffered formalin solution.

Nematodes were extracted from the sediment using a density gradient centrifugation in colloidal

silica (Heip et al., 1985). The fixed samples were rinsed using two sieves with different mesh. Each sample was first rinsed on a 1000 µm mesh sieve to separate shell detritus from the sediment, and then on a 38 µm mesh sieve. The fraction retained was well washed and centrifuged three times using the colloidal silica polymer LUDOX HS-40 (specific gravity 1.18 g cm^{-3}). The supernatant of each centrifugation cycle was collected, greatly washed on the 38 µm mesh and stored in a 4% formalin solution and rose Bengal. After the extraction completed, all nematodes were counted using a stereomicroscope Olympus DP70 (40x magnification) and a counting dish. From each replicate, a random set of approximately 120 nematodes was picked and transferred into a cavity box with a 99% formalin (4%) and 1% glycerol solution, to prevent the animals from collapsing. Cavity boxes were transferred into a sealed container with 95% (v/v) ethanol at 35ºC for approximately 12 hours. After this period, a couples of drops of ethanol (95% v/v) with glycerol (5% v/v) were added to the cavity boxes every 2 hours. Lastly, they were stored in anhydrous glycerol and mounted on slides (Vincx, 1996).

Nematodes were identified to genus level (Olympus BX50 light microscope and cell software D Olympus, Japan). Identification was made using pictorial keys (Platt & Warwick, 1988) and the online identification keys and literature from the Nemys database (Vanaverbeke et al., 2014).

Data analysis

The analysis performed (univariate and multivariate) aimed to detect temporal and spatial changes in the nematode assemblage such as digging/control plots and sampling occasions (T_0, T_1 and T_3). The statistical analysis of the biological and environmental data were performed in the PRIMER v6 software package (Clarke & Warwick 2001) with the PERMANOVA add-on package (Anderson et al., 2008).

Environmental data

Environmental data was analyzed through a Principal Component Analysis (PCA) in order to explore patterns in multidimensional data. PCA ordination reduces the number of dimensions with minimal loss of information and, in this particular case, was based on the values of each environmental variable (water content, salinity, organic matter, nitrate, nitrite, ammonium, phosphate, silt, clay and sand percentage and mean grain size) measured in control C_{11} and C_{18} plots and digging D_1 and D_{19} plots across each sampling occasion (T_0, T_1, T_3). The environmental

data's resemblance matrix based on Euclidean distance was performed. The data were also checked for uniform distribution and when necessary a log (X + 1) transformation was performed (ammonium, nitrate, phosphate, mean grain size and photosynthetic efficiency). All data were normalized subtracting the mean and dividing by the standard deviation.

Nematode assemblage

Nematode data from each control/digging plot and sampling occasion was made into a dataset in order to calculate, total nematode density (individuals 10 cm^{-2}), genera composition, trophic composition, ecological diversity indicators such as Margalef's richness Index (*d*) (Margalef, 1958), Shannon-Wiener diversity (*H'*) (Shannon & Weaver, 1963) and the genera Rarefaction (*EG*) (Hurlbert, 1971) and indicators based on ecological strategies as Index of Trophic Diversity (*ITD*) (Heip et al., 1985) and Maturity Index (*MI*) (Bongers 1990; Bongers et al., 1991).

The expected number of genera given by the rarefaction (*EG*) (Hurlbert 1971) was determined by the rarefaction curves, a procedure that scales down a collection of genera to the same number of individuals given by *E* (S_g) = $\sum_{i=1}^{s} \left[1 - \left(\frac{N-N_i}{n}\right) / \left(\frac{N}{n}\right)\right]$, where S_g is the total number of genera expected from a random sample of n individuals, drawn without replacement from N_i individuals distributed among i genera.

With the purpose to understand the trophic composition of the assemblages, feeding groups based on mouth morphology (Wieser 1953) were assigned to every nematode genus, going from selective (1A) and non-selective (2B) to epigrowth feeders (2A) and omnivores/predators (2B). Based on this information, it was possible to calculate the Index of Trophic Diversity (*ITD*) that is obtained by the sum of the squared proportional densitys of each feeding type (Heip et al., 1985), and its reciprocal (ITD^{-1}), so that the higher value obtained by the index correspond to the higher trophic diversity.

The Maturity Index (*MI*) is based in a life strategy measure, in which a value on a colonizer-persister scale (*c-p scale*) is assigned to each genus. This scale varies from 1 (colonizers) to 5 (persisters) (Bongers 1990; Bongers et al., 1991). Usually, rapid growth and reproduction rates characterize perfectly colonizers and these genera can also tolerate disturbance very well. Persisters, on the other hand, are characterized by slower growth and are often sensitive to non-stable ecosystems. This parameter is calculated as the weighted average of the individual colonizer-persister (*c-p*) scores as *MI* = $\sum_{i}^{n} = 1\ v(i) \times\ f(i)$, where $v(i)$ is the *c-p* of the taxon i and $f(i)$ is the frequency of that taxon.

The assemblage descriptors (total density, genera diversity, trophic composition and *d*, *H'*, *ITD* and *MI* indexes) were put through a three-way permutational analysis of variance (PERMANOVA) to test the null hypothesis that the nematode assemblages density, diversity and trophic composition does not significantly change between digging (D_1, D_{19}) and control (C_{11}, C_{18}) plots and among sampling occasions ("Time"). The PERMANOVA analysis was carried out following a three factor design: "Time": T_0, T_1, T_3 (3 fixed levels), "Treatment": Control and Digging (2 fixed levels) and "Plot (treatment)": C_{11}, C_{18}, D_1 and D_{19} (4 random levels). Nematode density data were square root transformed in order obtained a more balanced result in which the importance of the highly abundant genera are scale down in analysis of similarity between assemblages. The PERMANOVA analysis was conducted on a Bray-Curtis similarity matrix (Clarke & Green, 1988), where the null hypothesis was rejected at a significance level $p < 0.05$. When the number of permutations was lower than 150, the Monte Carlo permutation p was used. Whenever significant interactions in effects of the factors were detected, these were examined using a posteriori pairwise comparisons, using 9999 permutations under a reduced model. The similarity in assemblages in "Time", "Treatment" and "Plot (treatment)" were plotted by Principal coordinates analysis (PCO) using the Bray-Curtis similarity measure. The relative contribution of each genus to the dissimilarities between all factors was calculated using the two way-crossed similarity percentage analysis – SIMPER (cut-off percentage 100%).

In order to analyze and model the relationship between multivariate assemblage structure and environmental variables the DistLM (Distance Based Linear Model) was computed after checking for highly correlated variables that were excluded from the analysis. The analysis was performed after log (X + 1) transformation and normalized environmental variables using a sequential best procedure using the R^2 selection criterion for multivariate response variables (Anderson et al., 2008). A dbRDA (distance-based redundancy analysis) plot for the DistLM model was also created.

Results

Environmental data

Water content of the sediment ranged from 45.7% (plot D_1, T_3) to 64.2% (plot D_{19}, T_1) and reduction-oxidation potential (Eh) of the interstitial water varied from 183.1 mV (plot D_{19}, T_3) to 218.9 mV (plot C_{11}, T_0). Both control and digging plots generally presented a neutral pH (around 7) to slightly alkaline (7.6) (Table 1).

In general, organic matter (OM) content, ammonium concentration of the interstitial water of the sediment, mean grain size of the sediment and sand percentage were higher in the digging plots, all reaching higher values on plot D_{19}. On the contrary, nitrate, nitrite and phosphate concentrations and clay and silt percentage experienced a decrease in digging plots, with nitrate and nitrite showing minimum values at digging plot D_{19} while the phosphate lower value was found in digging plot D_1.

Table 1 Environmental variables measured *in situ* in control (C_{11} and C_{18}) and digging (D_1 and D_{19}) plots on each sampling occasion (T_0, T_1 and T_3).
Some nutrients' concentration is below the limit of detection (LD).

Environmental parameters	Control						Digging					
	Plot C_{11}			Plot C_{18}			Plot D_1			Plot D_{19}		
	T_0	T_1	T_3	T_0	T_1	T_3	T_0	T_1	T_3	T_0	T_1	T_3
WC %	56	52	50	54	47	51	52	49	50	53	51	52
Sal	34	35	38	37	34	37	35	37	38	36	34	40
T ºC	18.0	19.0	22.5	18.0	19.0	22.5	18.0	19.0	22.5	18.0	19.0	22.5
pH	7.2	7.2	7.5	7.4	7.6	7.2	7.3	7.6	7.5	7.2	7.6	7.6
Eh c	-21.0	-17.6	-30.5	-19.2	-35.8	-16.2	-18.8	-34.6	-35.5	-9.9	-38.1	-42.5
Eh mV	204.6	208.0	195.1	206.4	189.8	209.4	206.8	191.0	190.1	215.7	187.5	183.1
OM %	9	8	8	9	7	8	9	9	8	10	10	9
NH_4^+ µmol N/L	81.7	130.2	161.3	138.4	79.6	186.8	131.9	67.4	168.4	99.9	180.3	137.7
NO_3 µmol N/L	94.3	87.0	99.3	69.4	118.1	82.1	75.5	111.9	98.2	63.9	95.6	78.0
NO_2 µmol N/L	< LD	0.5	0.5	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD
PO_4 µmol/L	0.03	0.12	0.17	0.06	0.09	0.06	0.02	0.09	0.08	0.03	0.10	0.08
α	0.13	0.12	0.13	0.10	0.12	0.14	0.14	0.14	0.14	0.16	0.13	0.13
Mean grain size µm	103.5	126.0	110.6	116.0	97.7	89.3	122.4	131.6	120.3	134.2	99.6	98.4
Clay %	16.4	14.0	16.3	14.1	15.2	18.7	15.7	14.6	15.9	13.2	16.8	17.5
Silt %	43.9	43.4	47.7	43.9	46.5	47.7	42.6	40.5	46.2	41.9	45.2	47.1
Sand %	39.7	42.6	36.0	42.0	38.2	33.6	41.7	44.8	37.9	45.0	38.0	35.4
Gravel %	0	0	0	0	0	0	0	0	0	0	0	0

WC, water content; Sal, Salinity; T, Temperature; pH, potential of hydrogen; Eh, oxidation-reduction potential; OM, organic matter content; NH_4^+ - N, ammonium; NO_3^- - N, nitrate; NO_2^- - N, nitrite; PO_4^{-3} - P, phosphate; α, photosynthetic efficiency; mean grain size; clay < 0.004 mm; Silt 0.004-0.0625 mm; Sand 0.0625-2 mm; gravel > 2 mm.

The photosynthetic efficiency (α) of *Z. noltii* plants ranged from minimum values of 0.10 ± 0.02, in (plot C_{18}) to maximum values of 0.16 ± 0.01 (plot D_{19}), both at the beginning of the experiment (T_0). Plots were the turnover of the sediment was performed presented slightly higher photosynthetic efficiency (α) with exception of the plot D_{19}, at the third sampling occasion (T_3), where is possible to observe a decreasing of α (Fig. 3).

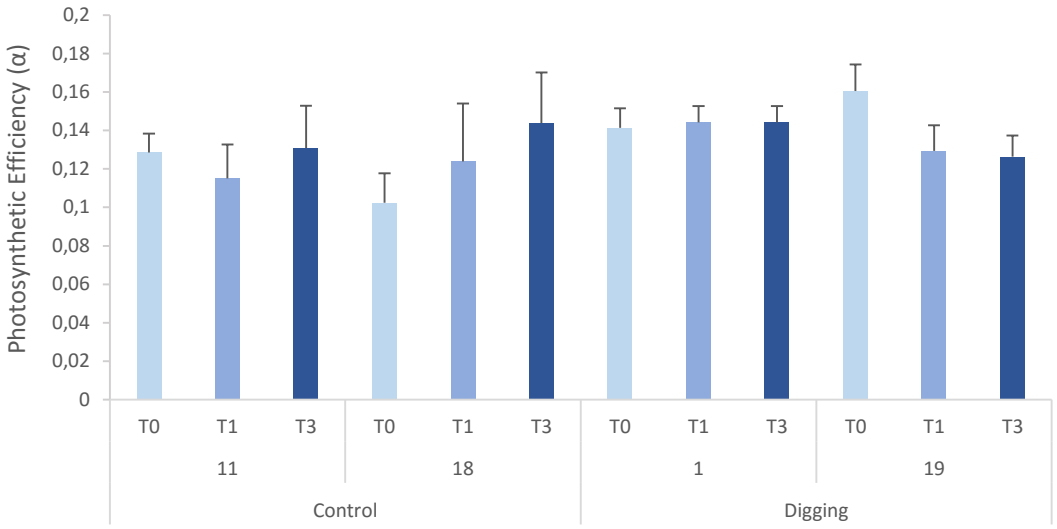

Fig. 3 Mean photosynthetic efficiency ± standard error (SE) of *Z. noltii* seagrass beds at each control (C_{11}, C_{18}) and digging (D_1, D_19) plot, on each sampling occasion (T_0, T_1 and T_3).

The PCA ordination of the environmental variables accounted for 44% (27% PCA1 and 17% PCA2) of the data variability. It is possible to observe the absence of a pattern and no clear separation between the control and digging plots was obtained (Fig. 4). However, it is possible to detect that organic matter and water content were higher in both control (C_{11}, C_{18}) and digging (D_1, D_{19}) plots, throughout the sampling occasions (T_0, T_1 and T_3). The mean grain size was higher in treatment plots at T_1 and nitrate explains control plots at T_1. Clay and silt proportions and ammonium concentration increased at T_3 in control plots.

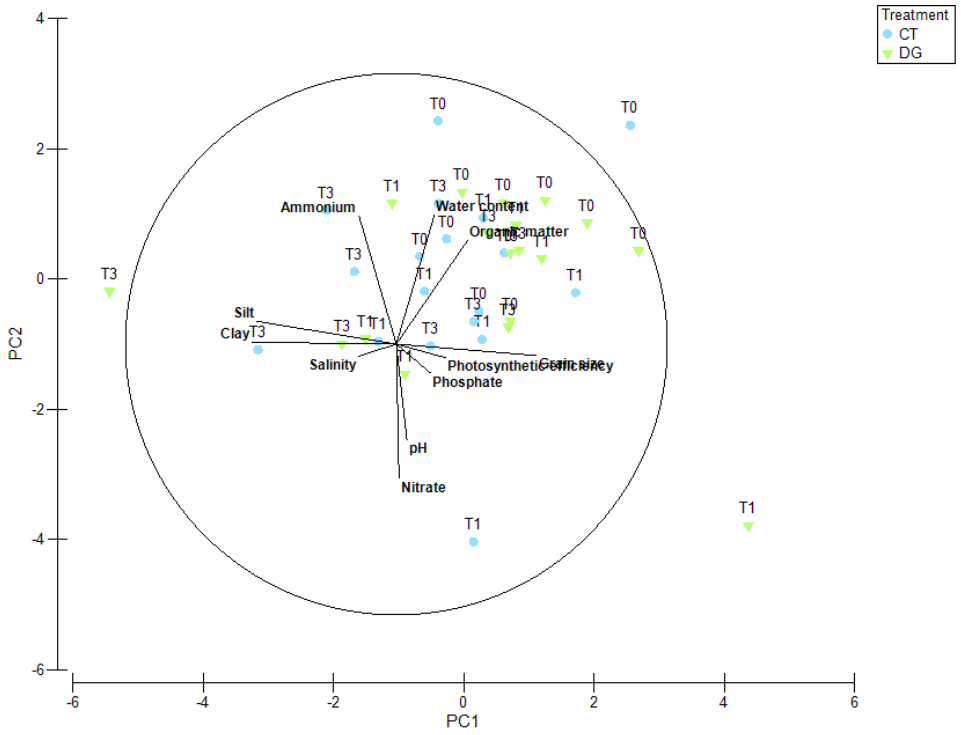

Fig. 4 Principal Component Analysis (PCA) plot based on the environmental variables measured in three sampling occasions ("Time": 3 levels, fixed), under control and digging treatments ("Treatment: 2 levels, fixed) performed in multiple plots ("Plot (treatment)": 4 levels, random). Vectors length corresponds to the correlation values. PCA1 = 27% and PCA2 = 17%

Nematode assemblages – density

Overall, nematode density varied from 599 to 8486 ind. 10 cm^{-2}. The treatment plots presented the mean density of 4958 ± 448 ind. 10 cm^{-2}, with minimum values in plot D_1 (3526 ± 1255 ind. 10 cm^{-2}), at the sampling occasion T_1 and maximum values in plot D_1 (6540 ± 1546 ind. 10 cm^{-2}), at the sampling occasion T_3. In the control plots the mean density was 3313 ± 166 ind. 10 cm^{-2}, with minimum nematode density found in plot C_{11} (1873 ± 967 ind. 10 cm^{-2}) and maximum densities in plot C_{18} (5264 ± 552 ind. 10 cm^{-2}). Both minimum and maximum values were found at the sampling occasion T_1, after de digging activity (Table 3). The plots C_{18} and D_{19} showed similar densities throughout the sampling occasions, while the plots C_{11} and D_1 were more similar (Fig. 5).

The PCO ordination for the nematodes densities did not reflect a distinct pattern between treatment and control plots. The low variability of the nematode density did not allowed to distinguish the control and treatment plots. Nevertheless, it is possible to identify that plot C_{18} and plot D_{19} had similar densities, changing almost the same way from T_0 to T_3 (Fig. 6).

PERMANOVA analysis for the nematode assemblages density showed significant differences (p

= 0.024) in the nematode densities between plots (Table 2), and the individual pairwise tests revealed that nematode densities are significantly different between plot D_1 to plot D_{19} (Pairwise Test. $p_{D1\ vs.\ D19} < 0.019$), although the control plots revealed no significantly differences (Pairwise Test. $p_{C11\ vs.\ C18} < 0.569$).

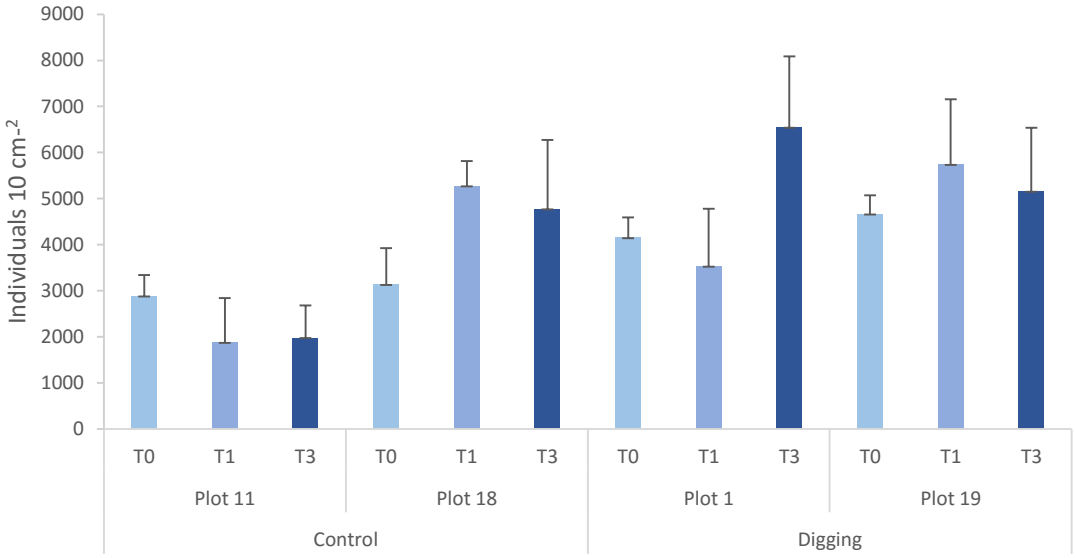

Fig. 5 Mean density ± standard error (SE) of nematodes (number of individuals per 10 cm^{-2}) at each control (C_{11}, C_{18}) and digging (D_1, D_{19}) plot, on each sampling occasion (T_0, T_1 and T_3).

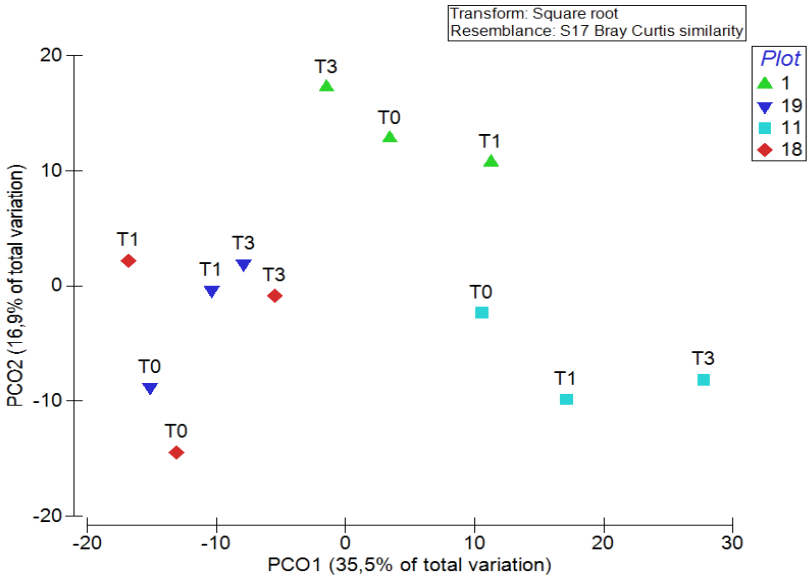

Fig. 6 Principal coordinates analysis (PCO) based on the nematode densities at each sampling occasion ("Time": 3 levels, fixed), under control and digging treatments ("Treatment": 2 levels, fixed) performed in multiple plots ("Plot (treatment)": 4 levels, random). PCO1 = 35.5% and PCO2 = 16.9%

Table 2 Three-factor PERMANOVA test with "Time" (3 levels, fixed), "Treatment" (2 levels, fixed) and "Plot" (2 levels, random and nested in "Treatment") for all variables analyzed. Bold values represent significant effects and interactions ($p < 0.05$).

	Source of variation	Degrees of freedom	Sum of squares	Mean Squares	Pseudo-F	pems	P (perms)	P (MC)
Nematode total density	Time	2	123.95	61.974	0.25441	999	0.782	0.868
	Treatment	1	1194.8	1194.8	1.6663	3	0.341	0.295
	Plot (treatment)	2	1434.1	717.04	4.1859	999	**0.024**	**0.017**
	Time x treatment	2	108.12	54.059	0.22192	998	0.815	0.866
	Time x Plot (treatment)	4	974.38	243.59	1.422	999	0.225	0.239
	Residual	24	4111.2	171.3				
	Total	35	7946.5					
Number of genera	Time	2	2157.3	1078.7	1.144	999	0.357	0.348
	Treatment	1	2071	2071	0.68182	3	0.668	0.744
	Plot (treatment)	2	6075	3037.5	3.8773	998	**0.001**	**0.001**
	Time x treatment	2	2036.9	1018.5	1.0802	997	0.397	0.397
	Time x Plot (treatment)	4	3771.4	942.85	1.2035	998	0.166	0.205
	Residual	24	18802	783.4				
	Total	35	34913					
Trophic composition	Time	2	302.95	151.47	0.39144	999	0.881	0.8709
	Treatment	1	766.79	766.79	1.0448	3	0.675	0.4534
	Plot (treatment)	2	1467.9	733.93	2.6888	999	**0.02**	**0.029**
	Time x treatment	2	770.66	385.33	0.99579	999	0.462	0.492
	Time x Plot (treatment)	4	1547.8	386.96	1.4177	998	0.175	0.189
	Residual	24	6551	272.96				
	Total	35	11407					
Margalef index	Time	2	486.94	243.47	3.8177	999	0.134	0.109
	Treatment	1	584.1	584.1	6.415	3	0.315	0.119
	Plot (treatment)	2	182.1	91.051	1.4139	998	0.279	0.266
	Time x treatment	2	12.605	6.3025	9.88E-02	999	0.927	0.939
	Time x Plot (treatment)	4	255.09	63.774	0.99028	999	0.409	0.41
	Residual	24	1545.6	64.399				
	Total	35	3066.4					
Shannon index	Time	2	353.23	176.62	11.281	997	**0.027**	**0.028**
	Treatment	1	2.1116	2.1116	1.98E-02	3	1	0.954
	Plot (treatment)	2	213.01	106.5	3.0002	998	0.074	0.065
	Time x treatment	2	107.59	53.794	3.4359	999	0.146	0.132
	Time x Plot (treatment)	4	62.626	15.656	0.44103	998	0.782	0.791
	Residual	24	851.99	35.5				
	Total	35	1590.6					
Index of Trophic Diversity	Time	2	67.997	33.998	1.2766	999	0.367	0.381
	Treatment	1	37.475	37.475	1.4838	3	0.335	0.353
	Plot (treatment)	2	50.511	25.255	0.98503	997	0.41	0.415
	Time x treatment	2	34.576	17.288	0.64913	998	0.559	0.587
	Time x Plot (treatment)	4	106.53	26.633	1.0388	999	0.435	0.391
	Residual	24	615.34	25.639				
	Total	35	912.43					
Maturity Index	Time	2	4.1716	2.0858	0.13137	999	0.891	0.879
	Treatment	1	0.49232	0.49232	1.68E-02	3	1	0.918
	Plot (treatment)	2	58.499	29.249	4.1716	999	**0.03**	**0.019**
	Time x treatment	2	24.026	12.013	0.7566	998	0.518	0.538
	Time x Plot (treatment)	4	63.511	15.878	2.2645	999	0.089	0.103
	Residual	24	168.28	7.0115				
	Total	35	318.98					

Table 3 Mean density ± standard error (SE) of nematode genera (number of individuals per 10 cm^{-2}) on control (C_{11}, C_{18}) and digging (D_1, D_{19}) plots on each sampling occasion (T_0, T_1 and T_3). Trophic group (TG) and *c-p* value of each genera included. Only the most abundant genera are included in this table.

			Control						Digging					
			Plot C_{11}			Plot C_{18}			Plot D_1			Plot D_{19}		
Genera	TG	*c-p*	T_0	T_1	T_3	T_0	T_1	T_3	T_0	T_1	T_3	T_0	T_1	T_3
Terschellingia	1A	3	695 ± 119	301 ± 81	291 ± 78	303 ± 107	2069 ± 240	1998 ± 1385	1348 ± 91	1376 ± 968	2443 ± 1170	656 ± 118	905 ± 278	1011 ± 502
Paracomesoma	1B	2	326 ± 69	440 ± 245	598 ± 200	225 ± 99	447 ± 48	675 ± 244	546 ± 127	320 ± 68	556 ± 288	1048 ± 150	978 ± 229	615 ± 438
Ptycholaimellus	2A	3	236 ± 7	214 ± 127	33 ± 17	457 ± 213	916 ± 523	203 ± 75	596 ± 85	371 ± 70	592 ± 33	462 ± 196	861 ± 200	450 ± 107
Linhomoeus	2A	2	247 ± 55	310 ± 214	414 ± 297	224 ± 69	103 ± 34	432 ± 308	500 ± 194	436 ± 161	306 ± 133	197 ± 36	596 ± 211	1151 ± 514
Sabatieria	1B	2	74 ± 35	133 ± 51	146 ± 61	358 ± 149	273 ± 85	313 ± 226	171 ± 66	241 ± 193	1181 ± 601	254 ± 209	563 ± 86	281 ± 256
Daptonema	1B	2	244 ± 18	126 ± 74	114 ± 36	336 ± 27	183 ± 70	164 ± 44	153 ± 28	76 ± 38	290 ± 123	353 ± 57	463 ± 150	380 ± 150
Odonthophora	2A	2	166 ± 53	93 ± 36	114 ± 46	237 ± 154	143 ± 36	170 ± 69	290 ± 131	193 ± 55	183 ± 55	276 ± 87	151 ± 68	291 ± 182
Metachromadora	1B	2	262 ± 192	19 ± 10	0	131 ± 52	73 ± 29	217 ± 149	14 ± 14	127 ± 44	206 ± 85	74 ± 8	110 ± 33	170 ± 66
Axonolaimus	1B	2	23 ± 12	50 ± 34	0	171 ± 81	179 ± 100	40 ± 12	14 ± 14	0	46 ± 23	162 ± 80	284 ± 157	0
Atrochromadora	2A	4	0	0	0	148 ± 82	206 ± 88	96 ± 78	0	0	0	344 ± 181	17 ± 17	140 ± 56
Anoplostoma	1B	2	7 ± 7	2 ± 2	0	30 ± 24	196 ± 67	104 ± 104	49 ± 9	57 ± 57	75 ± 43	117 ± 117	42 ± 42	45 ± 25
Metalinhomeus	1B	2	14 ± 14	13 ± 13	7 ± 7	147 ± 147	106 ± 106	33 ± 18	0	19 ± 19	50 ± 25	88 ± 88	0	156 ± 146
Paracyatholaimus	2A	2	10 ± 10	36 ± 21	9 ± 9	9 ± 9	79 ± 40	7 ± 7	36 ± 19	13 ± 13	0	100 ± 20	303 ± 131	17 ± 17
Sphaerolaimus	2B	3	14 ± 14	23 ± 9	33 ± 17	9 ± 9	103 ± 53	14 ± 14	14 ± 14	48 ± 34	81 ± 6	10 ± 10	25 ± 15	106 ± 40
Bathylaimus	1B	2	26 ± 15	10 ± 7	0	32 ± 26	44 ± 5	21 ± 21	56 ± 56	0	0	82 ± 26	59 ± 37	17 ± 17
Chromadorina	2A	3	30 ± 18	8 ± 8	31 ± 12	0	14 ± 14	0	39 ± 24	48 ± 34	141 ± 43	0	21 ± 21	0
Prochromadorella	2A	2	29 ± 5	13 ± 13	20 ± 18	45 ± 20	18 ± 18	21 ± 21	0	0	10 ± 10	95 ± 62	8 ± 8	61 ± 61
Microlaimus	2A	2	0	0	0	22 ± 11	30 ± 16	64 ± 44	13 ± 13	0	25 ± 25	15 ± 15	38 ± 19	104 ± 75
Halalaimus	1A	4	20 ± 11	4 ± 4	17 ± 8	37 ± 37	32 ± 16	20 ± 11	14 ± 14	6 ± 6	0	12 ± 12	108 ± 75	7 ±
Chromadora	2A	3	35 ± 22	0	2 ± 2	0	0	0	47 ± 29	54 ± 30	95 ± 66	15 ± 15	0	0
Synonchiella	2B	3	0	15 ± 12	9 ± 7	13 ± 13	0	0	11 ± 11	38 ± 38	102 ± 102	0	17 ± 75	0
Other genera			421 ± 2	65 ± 0.5	132 ± 1	194 ± 1	139 ± 1	172 ± 2	236 ± 2	102 ± 1	157 ± 1	294 ± 3	182 ± 1	150 ± 2

Nematode assemblages – structural diversity

Overall, 63 nematode genera from 23 families and 3 orders were identified from control and treatment plots. Both treatment and control plots presented similar percentages with most of the genera belonging to Monhysterida (52.5%), followed by Chromadorida (44%) and Enoplida (3.5%). The control plots presented a total of 53 genera with 90% of the assemblage being composed by Linhomoidae that had the higher representative percentage (39%), followed by Comesomatidae (21%), Chromadoridae (14%), Axonolaimidae (7%), Xyalidae (6%) and Desmodoridae (4%). In the treatment plots, 50 genera were registered, and as the control plots, Linhomoidea represented 38% of the assemblage, followed by Comesomatidae (23%), Chromadoridae (15%) and Axonolaimidae (6%), Xyalidae (6%) and Desmodoridae (3%). Together they comprise 90% of the assemblage. The remaining genera comprise only 5% of the assemblage.

In both control and treatment plots, *Terschellingia, Paracomesoma, Ptycholaimellus, Linhomoeus, Sabatieria, Daptonema* and *Odontophora* were the most abundant genera (Fig. 7), contributing for approximately 75% of the nematodes assemblages. All had higher densities in digging plots (Fig. 7). Overall, however, the higher diversity were primarily in control plots (C_{11} and C_{18}), only then followed by a treatment plot (plot D_{19}), as revealed by the rarefaction curve (Fig. 8). The SIMPER analysis gave information about the contribution of each nematode genera to the similarity values of the treatment and control plots. From de 63 genera of nematodes identified in this study, 40 genera were found in both control and digging plots whilst 23 genera were found in at least one of the groups. Genera *Terschellingia, Paracomesoma, Limonhoeus* and *Ptycholaimellus* contributed the most for the similarity within control and treatment plots but were also responsible for some of the biggest dissimilarities between them. *Cervonema, Quadricoma* and *Dichromadora* were important contributors for assemblage of the control plots but were absent from the treatment plots. *Eleutherolaimus* and *Theristus,* on the other hand, were absent from the control plots but contributed for the similarity in the plots that were under treatment (Table 4). Once again, PERMANOVA analysis for nematode assemblages structural diversity revealed significantly differences ($p = 0.001$) in the nematode assemblages between "Plot (treatment)" (Table 2). Individual pairwise tests revealed significantly differences within control plots (Pairwise Test. $p_{C11\ vs.\ C18} < 0.001$) and within treatment plots (Pairwise Test. $p_{D1\ vs.\ D19} < 0.003$).

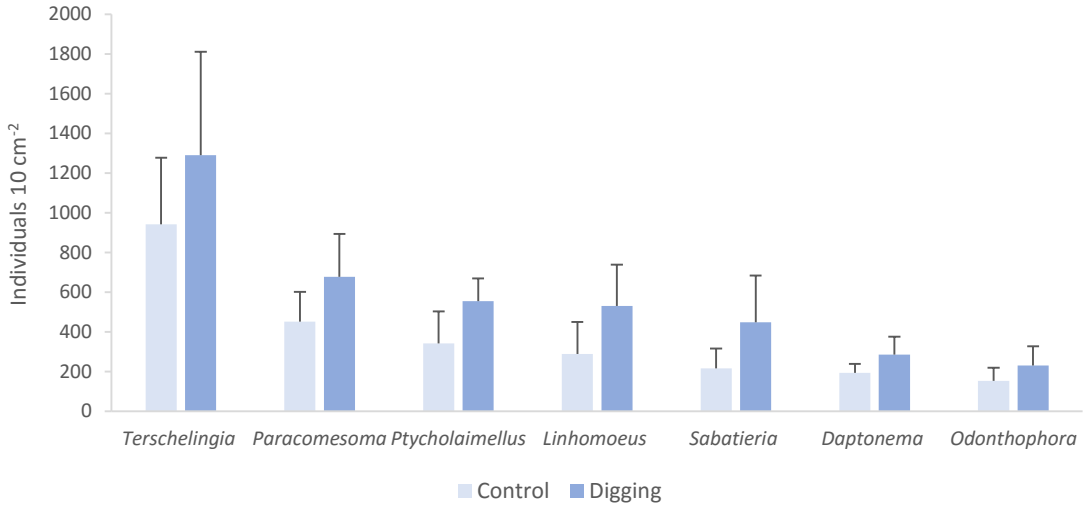

Fig. 7 Mean density ± standard error (SE) of the dominant genera at control and digging plot. The genera represented comprise approximately 75% of the nematode assemblages.

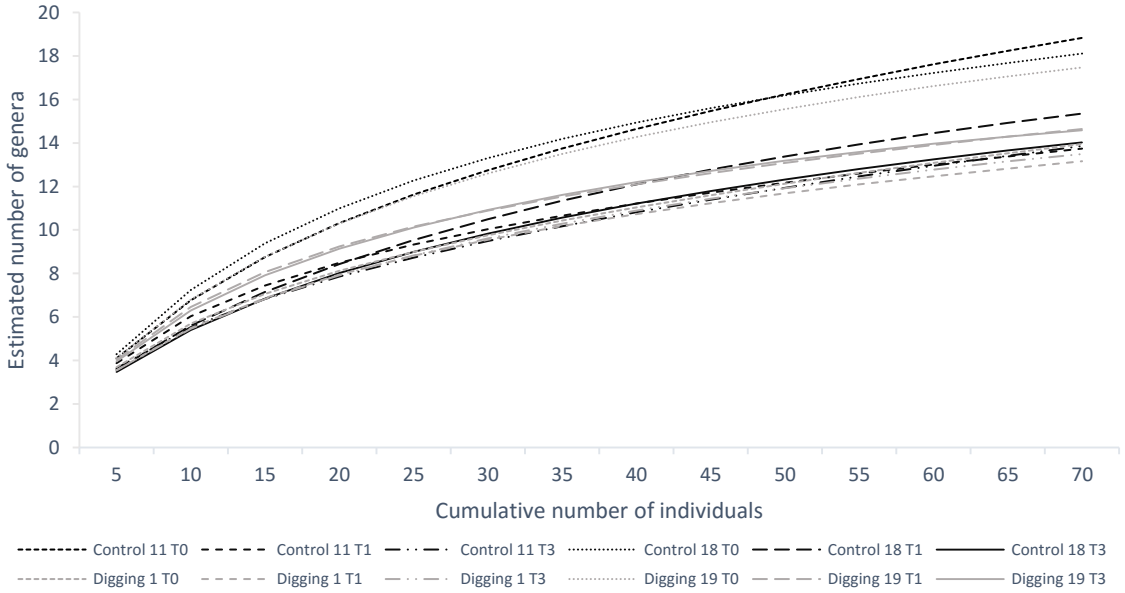

Fig. 8 Genera rarefaction curve (*EG*) for control (C_{11}, C_{18}) and digging (D_1, D_{19}) plots on each sampling occasion (T_0, T_1 and T_3).

Genera diversity based on Shannon-Wiener index (*H'*) registered the highest value at the sampling occasion T_0 (*H'* = 2.5) and the lowest value (*H'* = 1.9) at sampling occasion T_3, after 75 days of the beginning of the experiment, both in control plot C_{18} (Fig. 9). PERMANOVA analysis revealed significantly differences (p = 0.028) for factor "Time" (Table 2).

Nematode richness based on Margalef Index (*d*) registered the highest value in control plot C_{11}

(d = 2.4) at sampling occasion T_0 and the lowest value (d = 1.6) in treatment plot D_{19} at the sampling occasion T_3 (Fig. 9). PERMANOVA analysis for nematode richness revealed no significantly difference ($p > 0.05$) (Table 2).

For both diversity and richness index, pairwise tests revealed no significant interaction ($p > 0.05$) between factors “Time x Treatment” and “Time x Plot (treatment)” (Table 3).

Table 4 SIMPER analysis for the genera that contributed the most for the similarities and dissimilarities between control and digging plots.
Cut-off percentage of 100%

Genera	Control	Digging	Control vs. Digging	Genera	Control	Digging	Control vs. Digging
	Similarity		Dissimilarity		Similarity		Dissimilarity
	64%	68%	35%		64%	68%	35%
Terschelingia	13.59	16.61	8.27	*Cervonema*	1.64	0	1.89
Ptycholaimellus	8.05	11.55	5.14	*Setosabatieria*	0.16	0	1.66
Sabatieria	7.98	8.41	4.08	*Eleutherolaimus*	0	0.46	1.52
Atrochromadora	1.25	0.69	4.03	*Quadricoma*	0.33	0	1.44
Linhomoeus	9.91	9.94	3.91	*Comesa*	0.22	0	1.26
Axonolaimus	2.83	1.27	3.65	*Theristus*	0	0.18	1.18
Paracomesoma	12.72	12.05	3.45	*Dichromadora*	0.29	0	1.15
Metachromadora	3.38	4.34	3.22	*Calyptronema*	0.15	0	0.97
Anoplostoma	1.42	3.78	2.97	*Tricoma*	0.15	0	0.88
Daptonema	8.1	7.18	2.83	*Aegialoalaimus*	0.12	0	0.81
Metalinhomeus	2.69	1.32	2.78	*Descomolex*	0.13	0	0.73
Paracyatholaimus	2.13	1.76	2.65	*Oncholaimus*	0.15	0	0.64
Chromadora	0.07	1.23	2.51	*Cyartonema*	0.13	0	0.63
Chromadorina	1.03	1.24	2.29	*Nemanema*	0.11	0	0.52
Microlaimus	0.58	1.46	2.23	*Antomicron*	0.09	0	0.51
Bathylaimus	1.8	1.22	2.09	Other genera	< LD	< LD	9.01
Prochromadorella	2.85	0.77	2.04				
Synonchiella	0.51	0.91	1.93				
Odonthophora	7.48	7.57	1.91				
Sphaerolaimus	2.6	2.52	1.66				
Viscosia	0.37	0.93	1.6				
Halalaimus	2.39	1.06	1.59				
Molgolaimus	0,54	0,19	1,43				
Oncholaimellus	0,48	0,83	1,42				
Paracanthonchus	0,38	0,11	1,2				
Oxystomina	0,69	0,12	1,19				
Chromadorita	0,18	0,11	1,15				
Eurystomina	0,22	0,1	1,01				
Leptolaimus	0,13	0,1	0,97				

< LD, values below the level of determination

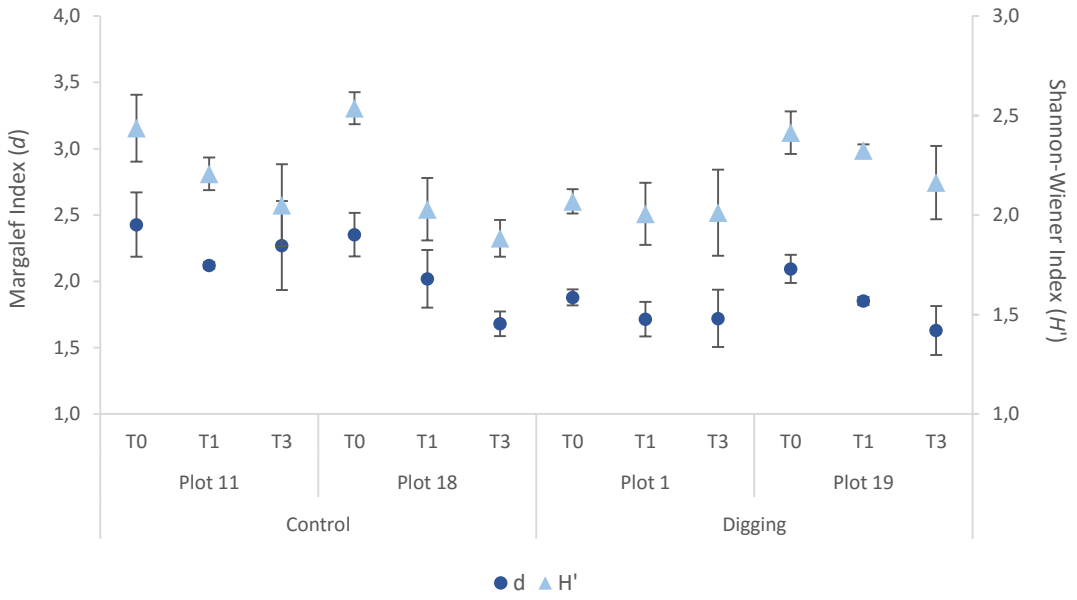

Fig. 9 Mean values ± standard error (SE) for Margalef Index (*d*) and Shannon-Wiener Index (*H'*) on control (C_{11}, C_{18}) and digging (D_1, D_{19}) plots on each sampling occasion (T_0, T_1 and T_3).

Nematode assemblages – trophic composition and functional diversity

In general, the dominance of trophic groups was similar for both control and treatment plots. The control plots were characterized mainly by epigrowth feeders (2A: 44 ± 12.3 %) and selective deposit feeders (1A: 33 ± 16.8 %), that encompassed approximately 77% of the nematode assemblage, with non-selective deposit feeders (1B: 22 ± 10.1 %) and omnivores/predators (2B: 1 ± 1.0 %) representing only 23% of the nematode assemblage. Similar results were obtained in the digging plots, with 75% of the assemblage being characterized by epigrowth feeders (2A: 45 ± 9.6 %) and selective deposit feeders (1A: 29 ± 1.0 %) and the remaining 25% of the assemblage comprising non-selective deposit feeders (1B: 24 ± 4.9 %) and omnivores/predators (2B: 1 ± 0.6 %) (Fig. 10). PERMANOVA analysis for the nematode trophic composition revealed significantly differences between "Plot (treatment)" ($p < 0.02$) (Table 2). Individual pairwise test showed that only the nematode assemblage between control plots was significantly different (Pairwise Test. $p_{C11\ vs.\ C18} < 0.015$) with the assemblage within digging plots being quite similar (Pairwise Test. $p_{D1\ vs.\ D19} > 0.215$). No significant interactions ($p > 0.05$) were showed between "Time x Treatment" and "Time x Plot (treatment)" (Table 2).

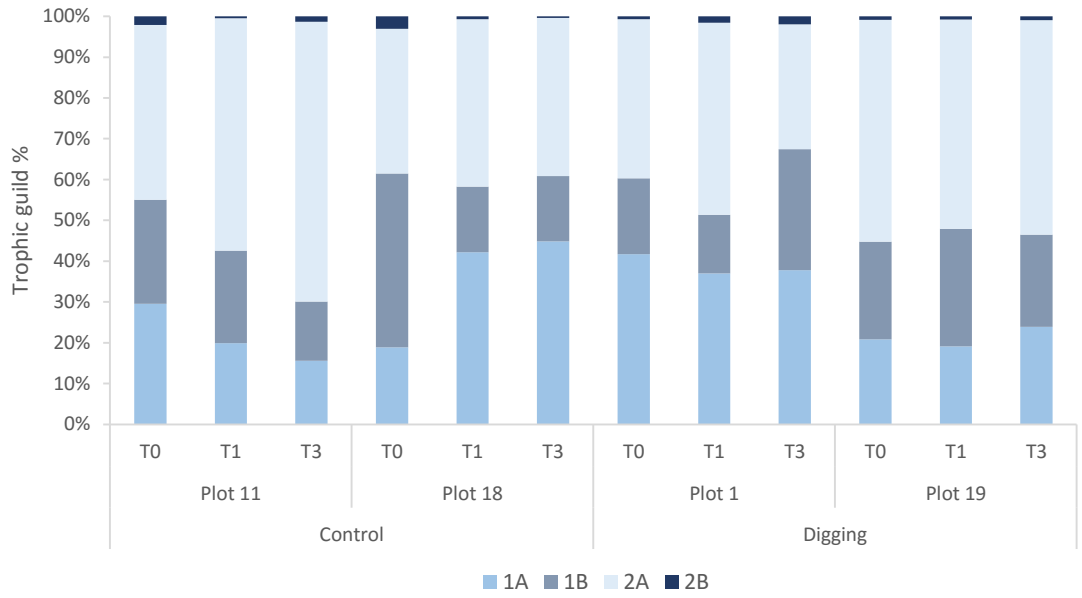

Fig. 10 Trophic guild composition (1A – selective feeders; 2A epigrowth feeder; 1B – non-selective feeders; 2B – omnivores/predators) on control (C_{11}, C_{18}) and digging (D_1, D_{19}) plots on each sampling occasion (T_0, T_1 and T_3).

Maturity index (*MI*) alternated from 2.3 ± 0.04 (plot C_{11}, T_3) to 2.7 ± 0.03 (plot C_{18}, T_1) in the control plots, and from 2.4 ± 0.02 (plot D_{19}, T_1) to 2.5 ± 0.1 (plot D_1, T_3) in the treatment plots. From the maturity index it was possible to observe that most genera belong in the colonizer category known as 'general opportunists' (*c-p* value 2), in both control (53%) and treatment (56%) plots, followed by the 'intermediate *c-p* group' (*c-p* value 3) that represented 42% of the assemblage, equally in control plots and treatment plots (Fig. 11). PERMANOVA analysis for the *MI* revealed significantly differences ($p < 0.03$) between "Plot (treatment)" (Table 2). Individual pairwise comparison on showed significantly differences within control plots (Pairwise Tests. $p_{C11\ vs.\ C18} < 0.01$) but not within treatment plots (Pairwise Tests. $p_{D1\ vs.\ D19} < 0.185$).

The index of trophic diversity (ITD^{-1}) ranged from 2.6 ± 0.06 (Plot C_{18}, T_0) to 3.1 ± 0.08 (plot C_{11}, T_0) in the control plots, and from 2.3 ± 0.02 (plot C_{18}, T_0) to 2.9 ± 0.18 (plot D_1, T_1) in treatment plots (Fig. 11). PERMANOVA analysis for the *ITD* indicated no significantly differences ($p > 0.05$) for nematode assemblages (Table 2).

Environmental variables *vs.* nematode assemblages

The DistLM analysis results revealed, as expected, that no environmental variable was significantly determinant ($p > 0.05$, for all variables) for the nematodes spatial and temporal distribution across control and treatment plots (Appendix, Table A1). Nonetheless, some

variables such as phosphate concentration, organic matter content and silt percentage might have created favorable conditions for the nematode assemblages (fig. 12).

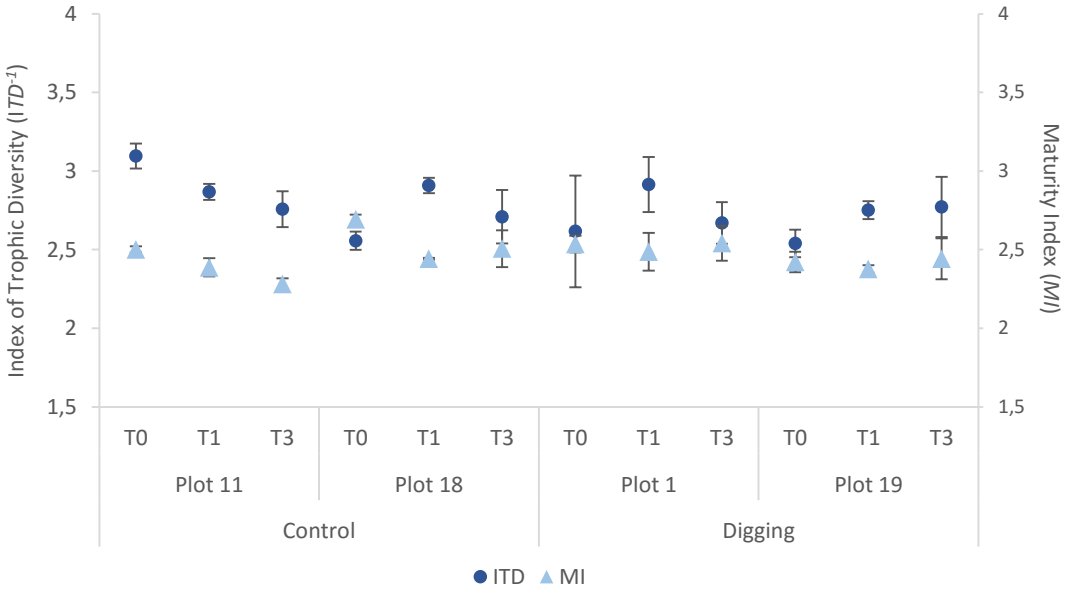

Fig. 11 Mean values ± standard error (SE) of the Index of Trophic Diversity (ITD^{-1}) and Maturity Index (*MI*) on control (C_{11}, C_{18}) and digging (D_1, d $_{19}$) plots on each sampling occasion (T_0, T_1 and T_3).

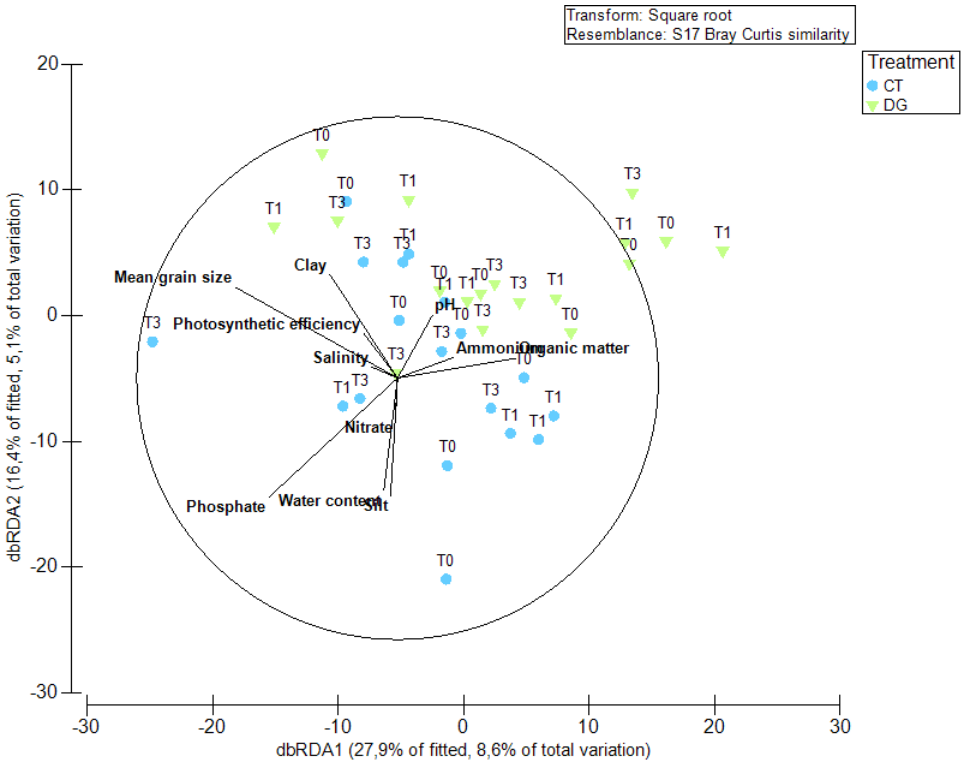

Fig. 12 Distance-based redundancy (dbRDA) plot illustrating the DistLM model (Best procedure; R^2 selection criterion) based on the nematode genera distribution on control (C_{11}, C_{18}) and digging (D_1, D_{19}) plots on each sampling occasion (T_0, T_1, T_3). dbRDA1 = 27.9% fitted; 8.6% total and dbRDA2 = 16.4% fitted; 5.1% total

Discussion

Meiofaunal assemblages provide information about the ecological conditions of an ecosystem, particularly those that have been disturbed, either naturally or anthropologically. In this particular case, we intended to understand to which point nematode assemblages of Mira estuary were affected by the bivalve harvesting activity, and its recovery in a habitat that is under recovery itself.

In general, the digging activity did not change substantially the nematodes density, either temporally or spatially, even though the highest values of genera richness and diversity were found on a control plot. It is possible to assume that the assemblages were able to recover to the turnover of the sediment in a short period of time (Dernie et al., 2003). Even though Kaiser et al., (2006) affirm that soft sediment biota of muddy sediments are vulnerable to disturbance, various authors found that meiofauna assemblages recover in months (Dayton et al., 1995; Kaiser et al., 2001; Dernie et al., 2003; Mirto et al., 2004; Dye 2006; Lee et al., 2011) or even within days or hours to its original rates in muddy sediment habitats (Warwick, 1986; Mistri et al., 2004; Johnson et al., 2007).

Grain size of the sediment is frequently linked with changes in the nematode density and control plots (T_3) had, in fact, higher proportions of silt and clay. Also, nematode density appear to be lower in plots where the grain size is superior to 125 μm, as meiofauna has some difficulty to move through the interstitial spaces of larger mean grain sizes (Coull, 1999). Soetaert et al. (2009) found a decreasing in the density of nematode assemblages when the sediment is coarser. The finer the sediment, the higher the range of microhabitats available for the nematode genera, which will result in an increasing of its diversity. Carvalho et al., (2013) found for macrofauna assemblages that higher diversity rates could improve the ecosystems capacity to recover after disturbance, which can also occur with meiofauna.

Likewise, the digging did not affect the *Z. noltii* seagrass beds as they maintained their photosynthetic efficiency, and its associated sediment did not change from control to digging plots nor from the beginning to the end of the experiment. Responsible for trapping and storing fine sediments, if the plants would have collapsed by the turnover of the sediment, coarse sediment would have taken a more prominent position within the substrata composition and thus the nematode density could have declined. Therefore, is it possible to assume that nematode evolution might not have been influenced by the bivalve harvesting.

As we intended to simulate the bivalve harvesting existent in the estuary, the raking performed at the beginning of the experiment did not penetrate very deep into the sediment. Furthermore,

the sediment was left *in situ* and its composition did not change, which could have helped with the nematode assemblage rapid recovery of the benthic infauna (Kaiser et al., 2001; Carvalho et al., 2013). For macrofaunal assemblages, Carvalho et al. (2013) found that sites whose tidal flats where bait harvesting was made by hand or using hoes/shovels recovered more rapidly than the ones using rakes or dredges, as the latter result in larger disturbed areas. The same happens when Johnson et al. (2007) studied the effect of crab-tilling in meiobenthic assemblages of mud-flats in Maine. In this experiment, although the sediment turnover was created by rakes, the disturbed sections were small which could explain the low negative effects of the physical disturbance of the sediment and the fairly quick recolonization. Besides, nematodes could have buried in the sediment. Schratzberger et al. (2004) believe vibrations in the sediment surface associated with harvesting activities (*e.g.* crab-tiling) may induce nematodes to burry below the sampling level both in treatment and, in a lesser extent, control plots near the place where disturbance took place, and to resurface afterwards. Johnson et al. (2007) consider that this could explain why the assemblage effects are not genera specific. Also, the little effect of the digging over the nematode assemblages might have occurred because this organisms inhabit dynamic environments as the estuaries, where they are usually in contact with natural stress, being regularly resuspended and transported by tidal currents (Sherman & Coull, 1980) and therefore being less sensitive to physical disturbance (Elliott & Quintino, 2007; Johnson et al., 2007; Elliott & Whitfield, 2011).

The genera obtained throughout this study are similar to other estuarine intertidal muddy sediments (Soetaert et al., 1995; Steyaert et al., 2007), particularly from the intertidal *Z. noltii* seagrass beds of the Mira estuary (Adão et al., 2009; Materatski et al., 2015; Materatski et al., 2016). Like in other estuaries, the seagrass beds of *Z. noltii* sediments presented a vast number of genera with only a few dominant: *Terschellingia and Linhomoeus* (Linhomoeidae), *Paracomesoma* and *Sabatieria* (Comesomatidae)*, Ptycholaimellus* (Chromadoridae)*, Daptonema* (Xyalidae) *and Odontophora* (Axonolaimidae) (Austen & Warwick, 1989; Soetaert et al., 1995; Adão, 2004; Johnson et al., 2007; Materatski et al., 2015; Materatski et al., 2016). In estuarine seagrass beds, the tidal currents and flows are likely to cause stress but particularly a hypoxic environment, to which these genera are often tolerant (Steyaert et al., 2007). Also, their small, motile bodies makes them capable of gliding through and over the fine sediments (Warwick, 1981). *Terschellingia, Paracomesoma, Ptycholaimellus* and *Sabatieria* densities*,* for instance, increased in the treatment plots. Together with their tolerance for hypoxic conditions, these genera increasing could be due to the slightly higher organic matter content observed after the digging activity. It has been reported that the turnover of the sediment might release buried organic

matter and nutrients to the surface that could have been responsible for the proliferation of some of the above mentioned genera (*e.g. Sabatieria in* Schratzberger & Jennings 2002).

Trophic composition results revealed that nematode assemblages evolved apart from the physical disturbance events, with no general pattern followed in control or digging plots. There is a predominance of epigrowth feeders (Ndaro & Olafsson, 1999; Materatski et al., 2015) and selective deposit feeders, and in a less extent, non-deposit feeders that is transversal to all plots. Usually, selective and non-selective deposit feeders diet consists primarily on diatoms, an important food source, and other microalgae, in accordance with the food pattern between these two trophic groups found by Vafeiadou et al. (2014) in a previous study of the Mira estuary trophic relations. Moens & Vincx (1997) have found that meiofauna individuals frequently have up to 40 and more diatoms in their intestine. The assemblages found in this study were composed by several genera with the above mentioned feeding ecology: *Paracomesoma, Daptonema, Sabatieria, Metachromadora, Axonolaimus* (epigrowth feeders), *Terschellingia* (selective deposit feeder), *Ptycolaimellus, Odontophora* and *Linhomoeus* (non-selective deposit feeders). Some of these genera such as *Ptycholaimellus, Paracomesoma* and *Daptonema* are, in fact, known for depending on a diatom diet (Moens & Vincx, 1998). Deposit feeders are usually opportunistic genera that can feed on various particles beside diatoms. Those food sources include bacteria and small detrital particles (Moens & Vincx, 1997). *Z. noltii* detritus and its associated micro-organisms, for once, are particularly important for the diet of *Ptycholaimellus, Daptonema* and *Metachromadora* and *Z. noltii* carbon inputs together with suspended particulate organic matter (SPOM) are used by genera such as *Sabatieria, Paracomesoma, Oncholaimus* and *Sphaerolaimus*. It is possible that the latter also feeds on *Terschellingia* (Vafeiadou et al., 2014). Moreover, epiphytic microalgae that are associated with seagrass beds might have a good nutritional value and were previously found to be an important food source for epistratum-feeding nematodes (Moens & Vincx, 1997; Vafeiadou et al., 2014). *Paracomesoma* and *Sabatieria* not only are good representatives of genera that can perpetuate in low oxygen conditions as they can feed on the high levels of organic matter and their associated bacteria (Soetaert et al., 1995; Schratzberger et al., 2006; Steyaert et al., 2007). Although selective deposit feeder are one of biggest trophic group in the nematode assemblages, it is important to notice that *Terschellingia* represent the single most abundant genus of this group. This genus is described as colonizers with great reproductive capacity, highly tolerant to extreme conditions (Moreno et al., 2011; Alves et al., 2013), usually present in hypoxic/anoxic muddy sediments, both in subtropical and tropical seagrass beds (Fisher et al., 2003; Adão 2004; Steyaert et al., 2007; Fonseca et al., 2011;

Materatski et al., 2015) and non-vegetated habitats (Materatski et al., 2015; Materatski et al., 2016).

In this study, the nematode diversity is observed by the presence and absence of some genera in control and treatment plots. Genera *Terschellingia, Paracomesoma, Linhomoeus* and *Ptycholaimellus*, for example, contributed the most for the similarity between treatments. Abiotic factors are normally related to the meiofauna composition, and in this particular case, no environmental variable was significantly different between treatments, which might explain the similarity of the meiofauna genera in both control and digging plots.

The index of trophic diversity (*ITD*) analysis result in no spatial or temporal patterns either on control and treatment plots and nematode assemblage maintained the four trophic groups in every plot. The higher ITD values represent high trophic diversity (Fonseca et al., 2011; Materatski et al., 2015), which are habitual in muddy and seagrass substrata. The maturity index (*MI*) presented low values, which was expected as seagrass beds typical maintain genera with 2-3 *c-p* value (Alves et al., 2013; Materatski et al., 2015; Materatski et al., 2016), representative opportunistic genera that dominate disturbed and polluted environments (Bongers & Bongers, 1998).

Although no medium or long-term negative effects resulted from this experiment, and the assemblage recovery was fairly quick, further research should focus on increasing the frequency of digging events as the turnover was performed merely once at the begging of the experiment. The permanent exposition to disturbance events may in fact promote an alteration in the nematode assemblages as a consequence of the habitat disruption.

General conclusion

In conclusion, this experiment indicates that in spite of the seagrass habitat disturbance caused by the digging activity, not only the nematode assemblages seemed to be resilient to physical disturbance related with bivalve harvesting as a “good environmental status” of the seagrass beds could be maintained. Also, the nematode assemblage presented the typical high density and diversity of the intertidal sediments of the seagrass beds, naturally adapted to high stress conditions.

Although nematode assemblages were able to recover quickly to the digging activity, further research is needed to comprehend the extent of their resilience, namely by increasing the intensity and frequency of the digging events.

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APPENDIX

Table A1 DistLM analysis for sequential best procedure using the R^2 selection criterion for multivariate response variables.

Environmental variable	SS (trace)	Pseudo-F	Prop.	p
pH	770,53	0,76731	2,21E-02	0,697
OM %	1095,8	1,1017	3,14E-02	0,317
WC %	757,28	0,75382	2,17E-02	0,701
NH4 µmol N/L	935,44	0,93605	2,68E-02	0,475
NO3 µmol N/L	643,56	0,63849	1,84E-02	0,821
PO4 µmol/L	1313,1	1,3287	3,76E-02	0,172
Clay %	907,21	0,90705	2,60E-02	0,511
Silt %	1001,3	1,0039	2,87E-02	0,403
Mean grain size	782,9	0,77991	2,24E-02	0,667
Sal	993,58	0,99593	2,85E-02	0,448
α	928,58	0,929	2,66E-02	0,502

pH, potential of hydrogen; OM, organic matter content; WC, water content; NH_4, ammonium; NO_3, nitrate; PO_4, phosphate; clay < 0.004 mm; Silt 0.004-0.0625 mm; mean grain size; Sal, Salinity; α, photosynthetic efficiency