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Research Article

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Spatial distribution patterns of microbiome and nematodes in response to sediment ecological conditions

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Abstract

Benthic organisms are crucial in the regulation of the ecosystem functions. They are primary mediators of the energy transfer in marine benthic food webs. Trophic interactions between benthic nematodes and sediment bacteria across divergent environmental conditions are poorly understood. The main goal of this study was to comprehend the relation between the spatial distribution patterns and diversity of benthic microbial communities and nematode assemblages of the intertidal sediments in Sado estuary (SW, Portugal). Samples were taken from three sites with different biogeochemical sediment conditions. Microbial communities were described using metagenomic approach, while nematode assemblages were characterized using morphological identification. Bacterial communities and nematodes assemblages presented significant heterogeneity between sites ($p<0.05$), which were primary related with the environmental variables (e.g., organic matter deposits and gravel percentage). However, while microbiome distributional patterns were clearly in accordance with ecological conditions of three selected sites (adjusted R2Adj = 0.53), nematode assemblages were more responsive to specific sampling locations within each site, suggesting that their response is rather driven by the within site specific factors, acting at the smaller spatial scale. Our study also demonstrated that the presence of Cyanobacteria (higher relative abundance of *Pleurocapsa* PCC-7319) seemed to be correlated with high abundance of nematode deposit feeders

Terschellingia and *Sabatieria* indicating possible interactions between these taxa. Our study represents a first insight into bacteria–nematodes associations underlying ecological conditions thereby providing an important baseline for the future understanding of the role that these two groups play in the benthic estuarine ecosystem.

Keywords: Sado estuary, microbiome, benthic nematodes, metagenomics

Introduction

Estuarine and coastal benthic ecosystems represent one of the major sources of essential services for human well-being [1, 2]. They play a crucial role in regulating fundamental ecosystem functions such as: food production, degradation and distribution of pollutants, recycling of nutrients and transfer energy through trophic processes [2]. These functions are mediated by intra and interspecific interactions between organisms that support the functional integrity of the benthic ecosystems [3].

Benthic nematodes represent 50-90% of biomass in shallow sediments [4] and are considered an important tool to assess the effects of natural and anthropogenic disturbances in marine and estuarine sediments [5, 6]. These organisms also play important roles in several ecosystem processes, being involved in complex relationships with microbial communities [1, 7]. Their buccal cavity morphology only allows the ingestion of a restrict type of food (e.g., bacteria or detritus) affecting the abundance and activity of the microbial communities [7, 8]. Furthermore, nematode activities related with bioturbation, extracellular polymeric substances (EPS) production and grazing have been proved to be an important contribute to stimulate the microbial development and growth [9–11]. Nematodes are thus important mediators of energy transfer to higher trophic levels [12–14], while sediment microbes are the primary facilitators of biogeochemical processes, such as carbon remineralization and sulphate reduction [15, 16]. A strong interconnection between nematode-microbe communities is well recognized, the presence of nematodes enhances microbial metabolic activities, while bacteria provide physiological adaptations to nematodes under hypoxic and anoxic conditions [8, 17, 18].

Assessing ecosystem conditions become one of the majors concerns over the past two decades. Majority of the studies have been focused on the analysis of a single taxonomic group distribution patterns and relate with ecosystem environmental parameters [19–21]. However, such approach does not consider the interaction between organisms belonging to different taxonomic entities, thereby limiting the assessment of the functional component of the ecosystem. The existence of the above-mentioned associations between bacteria and nematodes, still it is largely unknown how both groups interact in the context of community distributional patterns and most importantly if exists any congruence between both groups in their response to ecological conditions. Applying the novel high performance methodological approaches such as metagenomics to analyse the sediment microbiome diversity provide the possibility to develop essential understanding of the connection between benthic organisms. The main goal of this study was to understand the relation between the spatial distribution patterns and diversity of benthic microbial

communities and nematode assemblages of the intertidal sediments in Sado estuary in Portugal. The diversity patterns were investigated using: *i*) a metagenomic approach for microbial communities' assessment; and *ii*) a morphological approach for the characterization of nematode assemblages. The sediment biogeochemical conditions were analysed to assess the ecological conditions at each sampling site. Drawing from above it is hypothesized that spatial distribution patterns of microbial communities will follow the same patterns as spatial distribution of nematode assemblages, both responding to the biogeochemical variables of the estuary in a similar way.

Experimental Procedures

Study area and sampling design

The Sado estuary is the second largest estuarine system in Portugal, with an area of approximately 240 km^2, being one of the most important wetlands in Europe [22] (Fig. 1). The intertidal areas comprise approximately 78 km^2, of which 30% are salt marshes and intertidal flats [23]. Sado estuary has a semi-diurnal mesotidal system with tidal amplitude varying between 0.6 m and 1.6 m during spring and neap tides, respectively. Salinity is influenced by the Sado river flow (annual mean of $40 m^3.s^{-1}$) changing with seasonal and inter-annual conditions and temperature can range from 10 to 26°C.

The sampling sites were selected based on the expected differences in biogeochemical and trophic conditions of the sediments according to water hydrodynamics within an estuary (high/low water residence time), salinity gradient and the type of neighboring anthropogenic activities [24–26]. Based on above-mentioned criteria three "Sampling Sites" were selected: (1) Navigator Site located in the proximity of industrial area (Fig. 1). Main sediment fractions are dominated by fine sand and clay, with the high organic matter content while dissolved oxygen levels are reduced [27]; (2) Moinho Site is located within the borders of the Sado Nature Reserve, directly affected by the surrounding aquaculture activities. The aquaculture exploitation are the main contributors for the substantial high organic matter deposits, low dissolved oxygen concentrations levels and predominance of clay-like sediments [26]; (3) Tróia Site is located close to the estuary mouth, is directly exposed to the main estuary channel, contributing to high water exchange rate, high dissolved oxygen concentration levels and high proportion of the coarse type sediment (*e.g.*, sand) [26].

At each "Sampling Site", three sediment replicates were randomly collected at neap low tide to investigate microbiome communities, benthic nematodes assemblages, and measured environmental variables. The sediment physical-chemical composition was obtained analysing the granulometry (%)

(gravel, sand, and FF), elemental contents (W%) (OM, TN and TC, IN IC) and salinity (Supporting information Table S5).

Sample processing of benthic communities

Total DNA extraction of sediment and amplicon sequencing

Samples were taken from sediment surface at 10 cm depth into a sterilized 50-mL Falcon Tube, snap-freezed in dry ice and transported into the lab, where were stored at -80ºC until DNA extraction. Total DNA extraction from 0.25 g of the sediment was conducted under sterile conditions, using the MOBIO PowerSoil® kit (QIAGEN, Valencia, CA, USA) following the manufacturer's instructions. For cell lysis, samples were homogenized in a Precellys 24 Tissue Homogenizer (Bertin Instruments) for a total of 6 minutes (2x 20'' at 5000 rpm). The quality and quantity of total DNA was analysed through NanoDropTM2000 Spectrophotometer (Thermo Fisher) and Qubit4® fluorometer (Thermo Fisher Scientific). The presence of amplifiable DNA was confirmed by amplicon amplification with primers flanking the V4 region of 16S rRNA (515F–806R)[28]. A total of 9 samples were selected and sent for sequencing on Illumina MiSeq 2x 250bp (Illumina, Inc. San Diego, CA, USA) at EUROFINS Genomics (Cologne, Germany).

Bioinformatics analyses and data availability

Raw Illumina data was demultiplexed and quality-filtered using the defaults parameters of QIIME2 [29]. Single-end read data were denoised using DADA2 [30] plugin, that discarded biased reads (e.g., chimeras, singletons) and determined the amplicon sequence variants (ASVs). Further, ASVs were clustered into Operational Taxonomic Units (OTUs) at 97% similarity using VSEARCH open-reference OTU picking strategy against the SILVA v138 reference database [31]. Representative sequences were assigned taxonomy using a trained Naïve Bayes classifier (SILVA 138) [32] for V3-V4 hyper variable region from 16S rRNA. The resulting OTUs table was filtered to keep only features with a total abundance less than 10. OTUs classified as chloroplast, mitochondria, eukaryote, archaea, and unassigned were also removed. Filtered OTUs table was rarefied at 14000 sequences per sample after confirmation with rarefaction plugin. Raw data supporting our results have been deposited into the NCBI SRA repository under the Bioproject PRJNA680980 and accessions SRR13151077-13151079 (NAV), SRR13165305-13165307 (TRO), and SRR13165323- SRR13165325 (MOI).

Nematode assemblages

Nematode samples were collected at each sampling site by forcing a hand core (3.8 cm inner diameter) to a depth of 3 cm into sediment. Each replicate was fixed in a 4% buffered formalin. Each sample was first rinsed on a 1000 µm mesh sieve and then on a 38 µm mesh sieve. Nematodes were extracted from sediment using LUDOX HS-40 coloidal silica at specific gravity 1.18 g cm^{-3} [33]. Nematodes were counted using a stereomicroscope Leica M205 (100x magnification) and a counting dish. From each replicate, a random set of 120 nematodes was picked, transferred through a graded series of glycerol-ethanol solutions, stored in anhydrous glycerol, and mounted on slides for further identification [34]. Based on morphological characters, each nematode was identified until genus level (Olympus BX50 light microscope and cell software D Olympus, Japan). Identification was made using pictorial keys [35, 36], and online identification keys/literature available in the Nemys database [37].

Statistical analyses

The statistical analyses of the nematode assemblages and environmental data was performed using the PRIMER v6 software package [38] with permutational analysis of variance (PERMANOVA) add-on package [39]. Statistical analyses of 16S rRNA metagenomics was performed in Quantitative Insights into Microbial Ecology (QIIME2, version 2020.8) [29] and *phyloseq* R package [40].

Environmental factors

Environmental data was analysed using PCA. The analysis was applied to a data matrix based on three replicates from each "site" to explore patterns in multidimensional data. Data were log (X + 1) transformed (OM, %gravel and FF) and normalized [38]. The redundant variables were removed from the analysis (TN, TC and NO) and variables retained in the model acted as proxy for the ones that were eliminated (OM, gravel, sand, FF, IN, IC, CO and Sal).

Environmental factors influencing the communities

Redundancy Analysis (RDA) was conducted to test linear combinations of the environmental variables that best explain the variation of the microbial and nematode communities' patterns. The response dataset consisted of Hellinger-transformed relative microbial (observed OTUs with taxonomy assignment, corresponding to the 20 most abundant taxa) matrix, nematode genera abundance matrix [41] and explanatory environmental data. Variation inflation factors (VIF) where calculated to check for linear dependencies and to ensure that only variables with small VIFs (<10) were included. These were: "OM", "Gravel %", "FF_p", "IN_wp", "IC_wp" and "Sal". All the variables were transformed using arcsine

158 square root transformation, except for salinity that was log10 transformed. A forward selection procedure,
159 using function ordiR2step() was used to select only significant variables ($p < 0.05$) . RDA analysis was
160 performed in R [42] using "vegan" and "BiodiversityR" packages [43]. To test the correlation between both
161 ordinations (microbiome and nematodes) it was performed Pro- crustean test [44]. Procrustean test
162 measures the degree of concordance between two or more datasets having different characteristics and if
163 statistically significant, two datasets reflect in the same way the processes that determine their association
164 [45].

165 *Microbial communities*

166 α- and β-diversity indexes were calculated with q2-diversity plugin. For α-diversity analysis,
167 several descriptors were determined: Observed, Chao1, Shannon, Simpson and Pielou's Evenness. The
168 Kruskal-Wallis test, $p<0.05$ and Kruskal-Wallis (pairwise), were performed to determine the α-diversity
169 significance (within sites) for all groups and one-way PERMANOVA was performed to test null hypothesis
170 that no spatial significant differences between sites (β-diversity significance), $p<0.05$. The similarity of
171 microbial communities' composition in "Sites" were plotted by Principal Coordinates Analysis (PCO)
172 using the Bray-Curtis similarity. The relative contribution of each taxon to the dissimilarities between sites
173 was calculated using the two way-crossed similarity percentage analysis SIMPER (cut-off percentage
174 100%).

175 *Nematode assemblages*

176 Benthic nematode assemblages were characterized using the following parameters: total density
177 (individuals per 10 cm^{-2}) of the nematodes, genera, trophic composition and structural diversity (*e.g.*,
178 Margalef's richness Index (d) [46] and Shannon Wiener diversity (H_0) [47]. Indicators based on ecological
179 strategies were also calculated: Index of Trophic Diversity (ITD) [33] and Maturity Index (MI) [48, 49].
180 To assess the trophic composition of the assemblages, feeding groups based on mouth morphology were
181 assigned to every nematode genus [50], and the ITD was calculated [33]. The reciprocal index (ITD^{-1})
182 shows that the higher value of ITD^{-1} corresponds to the higher trophic diversity. The MI was used as a life
183 strategy measure, in which a value on a colonizer-persister scale (*c–p* scale) from 1 (colonizers) to 4
184 (persisters) was assigned to each genus. One-way PERMANOVA was performed to the assemblage
185 descriptors (number of genera, d, H, Trophic composition, ITD^{-1} and MI indices) to test the null hypothesis,
186 no significant differences of the density, diversity and trophic composition of the nematode were detected
187 between "Sites".

The PERMANOVA analysis were carried out with a one factor design: "Site": "Moinho"; "Navigator"; "Tróia" (3 levels, fixed) using a Bray-Curtis similarity matrix [38], $p<0.05$. The similarity of microbial and nematode communities' composition in "Sites" were plotted by Principal Coordinates Analysis (PCO) using the Bray-Curtis similarity measured. The relative contribution of each taxon to the dissimilarities between sites was calculated using the two way-crossed similarity percentage analysis SIMPER (cut-off percentage 100%).

Results

Environmental variables

The sampling area is delimited within the Sado estuary in three sampling sites – Moinho, Navigator and Tróia (Fig.1). The environmental variables measured in sediment and water in each site revealed contrasting conditions (Supplemental material Table S1) supporting a clear separation in the PCA (Principal Component Analysis) (PC1: 59.2% and PC2:31.4%) (Fig. 2). The spatial distribution patterns detected were mainly due to the highest values of sediment Organic Matter (OM), Organic Carbon (OC), Inorganic Nitrogen (IN) and Fine Fraction (FF) at Moinho site and the highest proportion of Gravel, Sand and Salinity (Sal) in Navigator and Tróia sites (Fig.2).

Bacterial communities

Sequencing statistics, diversity, and richness estimations

Illumina sequencing of the nine sediment samples (Navigator, NAVR1-R3; Tróia, TROIAR1-R3; and Moinho, MOIR1-R3) yielded a total of 435.781 sequence reads, out of which 175.796 high-quality V3-V4 16S rRNA sequence reads were clustered into 1.683 OTUs. For each sample, 15.000 reads were considered for further analysis after rarefaction (Supporting information Fig. S1). The observed OTUs were in accordance with Chao1 descriptor, obtained in all sites the following values: NAVR1-R3 (448-575), MOIR1-R3 (375-500) and TROIAR1-R3 (344-521) (Table S2). All sampling sites showed a high richness and diversity with Shannon index ranging between 7.8 (TROIAR3) and 8.56 (NAVR2), while Simpson Index and evenness were nearly 1. Further consideration of the α-diversity patterns, no significant differences ($p>0.05$) were obtained between bacterial taxa in each site nor between replicates within each site ($p>0.05$) (Table S3). In terms of β-diversity, based on PERMANOVA calculated at OTU level revealed a significant clustering of samples within the ordination space (between "Sites", $p<0.05$) (Table 1). The PCO showed a clear separation of bacterial community from Navigator site from other two sites (Moinho

and Tróia) (PC1 30.4% and PC2 17.5%) accounting together for app. 50% of the variability in the bacterial communities (Fig. 3).

Bacterial composition differences across sites

Bacterial communities from all sites are composed by 53 phyla from which 18 were the most representative, accounting for more than 90% of the taxa with more than 1% of the communities' total abundance (Fig. 4). The most abundant phyla were: Proteobacteria (38-42%), Desulfobacterota (20-23%), Chloroflexi (5-9%), Bacteroidota (5-6%) and Acidobacteriota (2%). In Moinho site, the bacterial community was composed by 247 genera from 269 families. The most abundant taxa belonged to the classes Gammaproteobacteria (38 genera), Alphaproteobacteria (30 genera), Bacteroidia (23 genera), Desulfobacteria (11 genera), Cyanobacteriia (8 genera), Anaerolineae (8 genera) and Desulfobulbia (7 genera). Within the most abundant classes (Gammaproteobacteria, Desulfobacteria and Desulfobulbia), the most representative orders were Steroidobacterales, Gammaproteobacteria_incertae_Sedis, Chromatiales, Desulfobulbales and Desulfobacterales, which were mainly represented by the families Woeseiaceae, Desulfobulbaceae and Desulfosarcinaceae, accounting for more than 20% of total abundance (Fig. S2). The most abundant genera were *Woeseia* (8.1-10.8%), *Sva0081_sediment_group* (4.3-5.6%), *Sva1033* (3.6-3.7%), *B2M28* (1.9-2.8%) and *Candidatus Thiobios* (1.6-2.5%).

In Navigator site, bacterial communities were composed by 321 genera and 306 families, whilst the major number of genera belonged to the classes Gammaproteobacteria (52 genera), Alphaproteobacteria (48 genera), Bacteroidia (31 genera), Desulfobacteria (10 genera) and Anaerolineae (10 genera). Within the most abundant classes (Gammaproteobacteria, Desulfobacteria and Cyanobacteriia), the most representative orders were Steroidobacterales, Desulfobacterales and Cyanobacteriales respectively represented by the families Woeseiaceae, Desulfosarcinaceae, Desulfobulbaceae and Xenococcaceae (Fig. S2). The dominat genera were *Woeseia* (8-9%), *Sva0081_sediment_group* (5.2-5.7%) and *Pleurocapsa*_PCC-7319 (0-4%), each one has a contribute >4% of the total density of the bacterial communities collected in Navigator site. The phylum Cyanobacteria were exclusively represented in abundance by the genera *Pleurocapsa_PCC-7319* at Navigator site. The Tróia bacterial community was composed by 249 genera and 272 families, whereas the most abundant taxa belonged to the classes Alphaproteobacteria (36 genera), Gammaproteobacteria (34 genera), Bacteroidia (26 genera), Desulfobacteria (9 genera), Desulfobulbia (8 genera), Anaerolineae (11 genera), Cyanobacteriia (3 genera) and Acidimicrobiia (4 genera). Within the most abundant classes (Gammaproteobacteria and

247 Desulfobacteria), the most representative orders were Steroidobacterales and Desulfobacterales
248 (Woeseiaceae and Desulfosarcinaceae families), counting more than 17% of total abundance of the
249 community (Fig. S2). The most representative genera of communities were *Woeseia* (6.9-8.1%),
250 *Sva0081_sediment_group* (3.7-7.3%), *SEEP-SRB1* (1.7-2.6%), *SBR1031* (1.8-3.7%) and *Sva1033* (2.7-
251 3.6%) each one corresponds to >1% of Tróia communities' total abundance.

252 The SIMPER analysis showed that bacterial taxa that most contributed for the similarity within
253 sampling sites were the *Woeseia*, *Sva0081_sediment_group* and *Sva1033* (Similarity ≥68 %). Moreover,
254 the great contributors for the major dissimilarities between sites were the genera *SEEP-SRB1* and *SBR1031*
255 (Moinho vs Tróia, dissimilarity 29.94%), *Pleurocapsa_PCC-7319, Myxosarcina_GII* and
256 *Cyanobacterium_CLg1* from the order Cyanobacteriales (Moinho vs Navigator and Navigator vs Tróia,
257 dissimilarity 34-36%).

258 **Nematode assemblages**

259 *Density and structural diversity*

260 Overall, the nematode density varied between 2706.3 and 13466.9 individuals per 10 cm^{-2} (Table
261 2). The nematode assemblages of Moinho site registered the highest mean density (13466.9 ± 1631.1 ind.
262 10 cm^{-2}), whilst the lowest mean density was obtained at Navigator sampling site (2706.4 ± 1092 ind. 10
263 cm^{-2}). PERMANOVA analysis for the nematode density revealed significant differences between "Sites",
264 $p \leq 0.05$ (Table S4). Nematode assemblages' diversity was high in all sampling sites (Table S5). Nematode
265 richness based on Margalef Index (*d*) registered the highest value in "NAVR3" (*d*=2.86) and lowest values
266 was registered in "MOIR1" (*d*=0.86). Genera diversity based on Shannon-Wiener index (H′) registered the
267 highest value at "NAVR3" (H'=2.29) and the lowest value at "MOIR1" (H'=1.2) (Table S5).
268 PERMANOVA analysis conducted based on structural diversity descriptors (d, H' ITD^{-1} and MI)
269 demonstrated significant differences between "Sites", $p \leq 0.05$, only for Margalef richness (d) (Table S4).

270 The nematode assemblages collected in the Navigator site were composed by 29 nematode genera
271 belonging to 14 families. Most of the genera belonged to the orders Monhysterida (46.6%), Chromadorida
272 (26.8%) and Enoplida (6%) and the dominant families were Linhomoeidae (53.3%), Desmodoridae (15.9%)
273 and Comesomatidae (10.8%). The genera *Terschellingia* (52%), *Metachromadora* (15%), *Sabatieria* (9%)
274 and *Anoplostoma* (5%) represent 82% of the total density of the nematode assemblage collected in
275 Navigator (Fig. 5 and Fig. S3). The remaining 25 nematode genera accounted 18% of the total density. The
276 assemblages that accounted for the highest density values were from Moinho sampling site, they were

277 composed by 20 nematode genera belonging to 13 families. Most of the identified taxa belonged to the
278 orders Chromadorida (59.2 %), Monhysterida (20 %) and Enoplida (1.8%), being the dominant families
279 Desmodoridae (56.1%), Linhomoeidae (13.8%) and Comesomatidae (8.8%). *Metachromadora* (56%),
280 *Terschellingia* (14%), *Sabatieria* (7%) and *Axonolaimus* (5%) and they accounted for 84% of the Moinho
281 assemblages (Fig. 5 and Fig. S3). The nematodes identified at Tróia sampling site were composed of 20
282 genera from 12 families. Almost all genera belonged to the orders Chromadorida (42.5 %), Monhysterida
283 (12.3%) and Enoplida (2.5%) with the dominant families being Desmodoridae (56.1%), Linhomoeidae
284 (13.8%) and Comesomatidae (8.8%). The 4 dominant genera *Metachromadora* (49%), *Terschellingia*
285 (16%), *Ptycholaimellus* (7%) and *Sabatieria* (7%) represent 80% of the assemblages collected in Troía site
286 (Fig. 5 and Fig. S3).
287 The PCO ordination of the nematode assemblages showed a clear separation of Moinho site from
288 Navigator and Tróia sites. These results reflect high variability of nematode density between sites (PC1
289 40.9 % and PC2 25% contributions) together accounted for about 65.9 % of the total variability in the
290 density data (Fig. 6). Concerning this ordination, SIMPER analysis showed that *Metachromadora*
291 *Terschellingia, Sabatieria* and *Axonolaimus* were the genera that most contributed for the similarity within
292 sites, while *Metachromadora Terschellingia* and *Axonolaimus* were the genera that most contributed for
293 the dissimilarity between sites. Although the assemblages of Moinho and Navigator sampling sites were
294 clearly separated, the nematode community profiles showed distinct spatial distribution patterns comparing
295 to those obtained for the microbial communities.
296 *Trophic composition and Functional diversity*
297 Navigator assemblages were mainly characterized by selective deposit feeders (1A) and non-
298 selective deposit feeders (1B), representing 63% of the nematode assemblages. While 52% of the Moinho
299 assemblages were characterized by omnivores/predators (2B). Tróia nematode assemblages were mainly
300 comprised of omnivores/predators (2B: 45%) and selective deposit feeders (1A: 18%) (Table 2).
301 PERMANOVA analysis of the nematode trophic composition data revealed significant differences between
302 "Sites" ($p \leq 0.05$) (Table S4).
303 The Trophic diversity Index (ITD^{-1}) values ranged from 1.7 to 3 where the highest value was
304 obtained at Navigator site (varying from 2 to 3), corresponding to the highest trophic diversity between
305 assemblages of each site, while the lowest values (varying from 1.7 to 2.3) was registered in Moinho (Table
306 S5) besides the highest density values at this site. The maturity index (MI) varied from 2.1 to 2.7 and the

highest values were obtained in Navigator sampling site, while the lowest values were registered in Tróia. These results revealed the colonizer strategy (*c-p* value 2) dominated at Moinho (57%) and Troia (42%). Navigator sampling site was dominated (46%) by the genera classified as *c–p* value 3. PERMANOVA analysis of the functional diversity descriptors revealed no significance differences between "Sites" ($p \leq 0.05$) (Table S4), highlighting the prevalence of similar trophic diversity and opportunistic strategies among nematodes inhabiting all three sites.

Environmental factors influencing both benthic communities

The RDA ordination on microbial communities constrained by the environmental variables was highly significant (F =5.44, p = 0.001, adjusted R2Adj = 0.53) (Fig. 7A). The cumulative explained proportion of both axes was remarkably high reaching 64.44%. The environmental variables that emerged as significant were % Gravel (p=0.045) and % OM (p=0.005). According to triplot (Fig. 7 A), there was a very clear separation of all sites, particularly Navigator with clear separation from Moinho and Tróia along the first axis accounting for the highest proportion explained (56.27 %) of the total variability in community data. High proportions of gravel in NAVR3 were correlated with the prevalence of Xenococcaceae family. Desulfocapsaceae, Ilumatobacteraceae and BD2-11_terrestrial_group were associated to NAVR1 and NAVR2. Moinho was characterized by high OM deposits with certain affinity of: Desulfobulbaceae, B2M28, Chromatiaceae and Sva1033 families. Tróia sampling sites (TROIAR1-R3) were tightly grouped together with strong affinity of SBR1031 and Desulfosarcinaceae families as well as Cyclobacteriaceae (Fig. 7A).

The RDA ordination on nematode assemblages' data constrained by the environmental variables was highly significant (F =2.49 p = 0.001, adjusted R2Adj = 0.35), with the first RDA axis explaining 32.82% and the second explaining 19.91% of the variance in nematodes assemblage data (Fig. 7B). Significant emerged environmental variables were: % Gravel (p=0.035), % OM (p=0.015) and inorganic carbon (p=0.010). Similarly, as it was observed with microbiome communities Navigator was separated from the remaining sites. This separation was driven by the high % Gravel content at NAVR2 and Inorganic Carbon at NAVR1. Genera associated to NAVR2 included *Odontophora*, *Calyptronema*, *Aegialoalaimus* and *Metalinhomoeus*, whereas *Terschellingia*, *Microlaimus*, *Camacolaimus* and *Spirina* were instead associated to NAVR1. The presence of *Viscocia* and *Paracomesoma* were highly related to NAVR3. On the other hand, *Metachromadora* was demonstrated to yield clear affinity to TROIAR2 and MOIR1, while the presence of *Axonolaimus* was highly correlated with MOIR2 and TROIAR3.

Besides microbial-based RDA had only two environmental variables significantly correlated with the community ordination, its overall significance and remarkably high AdjRsquare indicates that only these two variables were able to predict majority of the variability occurred in microbial community data. Additionally, only 20 most abundant microbiome families were sufficient to clearly separate 3 distinct sites. On the other hand, nematode assemblages were more responsive to specific sampling replicates within each site. Organic matter and gravel were demonstrated to yield equal importance (yet exhibited in a different way) for the distribution patterns of both bacterial and nematode groups. The result of Procrusetan test that analysed the correlation between both ordinations was not significant (Correlation in a symmetric Procrustes rotation: 0.4897, p=0.324).

Discussion

The high spectrum of environmental conditions registered in estuarine sediments are well known to capture a variety of adaptive responses in benthic communities [27]. In this study, the spatial distributions of microbial and nematode communities were studied in three contrasting sites of Sado estuary. As initially hypothesized, both benthic communities followed a consistent distribution pattern, being congruent with the sediment biogeochemical conditions.

The influence of the environmental conditions on the spatial distribution patterns of the bacterial communities is well known [51, 52]. At all sites, microbial communities were dominated by Proteobacteria and Desulfobacteriota phyla, both known to play important roles in most of the biogeochemical processes (e.g., anaerobic processes in sulfur and carbon cycles) in estuarine sediments [52–54]. The presence of Woeseiaceae and Halieaceae families were also detected in all sampling sites, which is corroborated by their wide occurrence and contribution to the biogeochemical processes in marine sediments [55, 56]. Symbiotic organisms from the family Rhodobacteraceae [11] and sulfur-oxidizing bacteria *Candidatus Thiobios* were also detected in abundance in all sampling sites. These organisms are recognized to be involved in symbiotic mechanisms with nematodes, providing them beneficial physiologic adaptations (such as protection against adverse conditions) [17, 57, 58]. In the case of Moinho site, the high prevalence of Desulfobulbaceae family may evidence the anoxic conditions of the sediments, since the metabolic processes of these sulphate reducers are mainly involved in anaerobic degradation of organic matter [54, 59]. Cyanobacteria were responsible for separating bacterial communities of Navigator from the other sites, with the exclusive presence of the unicellular and pseudo-filamentous genus *Pleurocapsa* [60]. These organisms are regarded as ecological important group in estuarine and coastal environments being primary

367 producers and nitrogen/carbon fixators, and their increased grow highlight the presence of opportunistic
368 species with the release of cyanotoxins [60, 61]. The distribution patterns of bacterial communities at the
369 Navigator site, highlighted by the exclusive presence of cyanobacteria, are strictly related to the high
370 proportions of gravel and salinity, which is in accordance with Kolda [60], that showed the preference of
371 cyanobacteria for a sandy gravel type of sediment.

372 As for the nematode communities, their density was similar to those reported other Portuguese
373 estuaries (SW coast of Europe), such as Mira, Mondego and Sado estuaries [19, 27, 62] with the exception
374 of Moinho sampling site that diverged from others registering the highest nematode density. The highest
375 content of OM at this site could be related, since nematode density and distribution are highly related to the
376 food and OM available at the bottom of the sediments [9, 63]. The predominance of sandy sediments in
377 Navigator and Tróia sampling sites contributed to the low nematode density but a diversity increase,
378 possibly related with the broader range of microhabitats available for nematodes in these sediments when
379 compared to muddy ones [64]. In all sampling sites, the dominant genera were *Metachromadora,*
380 *Sabatieria, Axonolaimus* and *Terschellingia,* similar composition to mud-flat areas of Mondego estuary
381 [62] and also to the previous study in Sado estuary [27]. The spatial distribution patterns base on $ITD^{-1}$and
382 MI indexes were also similar to those verified in Mondego, Mira and Sado estuaries [19, 27, 65]. However,
383 the trophic composition of nematode assemblages showed different results from previous studies (i.e.,
384 usually dominated by non-selective deposit feeders (1B) and epistrate feeders (2A) [20, 62]. The
385 omnivores/predators (2B) were dominant in all sampling sites, which are mainly grazers of
386 microphytobenthos and bacteria [66]. These organisms are usually able to vary their feeding mode in
387 response to the food availability being difficult to draw a general trend in abundance of these feeding groups
388 [9, 12]. Nematode communities sampled in Navigator showed a high percentage of non- and selective
389 deposit feeders (1A and 1B) such as *Terschellingia* and *Sabatieria,* which are usually favoured by the
390 depositional nature and hypoxic conditions of the sediment. Moreover, Vafeiadou [12] and Sahraean [67]
391 demonstrated by stable isotope analysis that *Terschellingia* can thrive under conditions that benefit the
392 chemoautotrophic prokaryotic activity (e.g., *Cyanobacteria*) by using methane-derived carbon as energy
393 source. Schuelke [68] did not find a clear distribution patterns of nematodes-associated microbiomes
394 between different geographical areas with different environmental conditions. However, the dataset did
395 reveal a variety of new ecological interactions, including putative symbionts and parasites as well as
396 associations with prokaryotic taxa involved in methane and nitrogen cycling. The type of nematode-bacteria

interaction is not possible to be defined by our results, yet the abundance of *Terschellingia* and Cyanobacteria at Navigator sampling site may suggest a possible connection between benthonic organisms considering the previous studies [12, 67].

Concluding remarks

Our study demonstrated that nematodes assemblages and microbiome communities respond closely to the ecological conditions of the estuary. In fact, the RDA analysis showed that, for both benthic communities, the OM and the %Gravel were the main contributors of their spatial distribution patterns in two of the sampling sites. These findings yield great potential and opens a new avenue for the inclusion of the microbiome in the future ecosystem health assessments. This study introduces a new basis for the future development of the specific methods and tools to analyse the interactions existing between nematodes and microbiome as well as understanding the role that microbiome has in mediating the sediment-nematodes relationship in marine benthic ecosystems.

411 **Statements & Declarations**

412

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422

423 **Author Contributions**

424 Cláudia S. L. Vicente and Helena Adão contributed to the study conception and design. All authors
425 contributed for the sampling collections. Sediment analysis were performed by Joana Neves, Marta Martins
426 and Helena Costa. Data collection and analysis were performed by Soraia Vieira, Kasia Sroczyńska, Helena
427 Adão and Cláudia S. L. Vicente. The first draft of the manuscript was written by Soraia Vieira, and all
428 authors commented on previous versions of the manuscript. All authors read and approved the final
429 manuscript.

430

431 **Data Availability**

432 The data sets generated and analysed during the current study are available in NCBI SRA repository under
433 the Bioproject PRJNA680980 (https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA680980) and
434 accessions SRR13151077-13151079 (NAV), SRR13165305-13165307 (TRO), and SRR13165323-
435 SRR13165325 (MOI).

436

437 **Code availability**

438 Not applicable.

439

440 **Ethics declarations**

Not applicable

Consent for publication

Not applicable

Conflict of interest

The authors declare no competing interests.

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640 **Figures captions**

641

642 **Figure 1**. Sado estuary located at Southwest of Portugal (38° 31′ 14″ N, 8° 53′ 32″ W). The selected
643 sampling sites: Navigator (38.487033, -8.795686) (grey circle), highly industrialized area; Moinho
644 (38.528101, -8.802995) (orange circle) with high organic inputs and Tróia (38.417317, -8.816433) (green
645 circle) with coarser sediment. Moinho and Tróia are situated in a protected area

646

647 **Figure 2.** Principal component analysis, based on Bray-Curtis Distances, according to environmental
648 variables measured (OM, gravel, sand, FF, IN, IC CO and salinity) at each sampling site: Navigator,
649 Moinho and Troia, PC1 59.2%; PC2 31.4%

650

651 **Figure 3.** Principal component analysis, based on Bray-Curtis Distances, according to OTUs relative
652 abundance of genera level obtained in each "site". PC1 = 32.6 % and PC2 =17.4 %. The vectors are the
653 most representative genera of the variability observed in bacterial communities

654

655 **Figure 4.** Bar plot displays the relative abundance of OTUs (%), based Phylum-level, that represents more
656 than 90% of the total abundance in each site Moinho (MOI); Navigator (NAV) and Tróia (TROIA), n=3,
657 the relative frequencies smaller than 1% collapsed into the N/A category

658

659 **Figure 5.** Bar plot displays the highest density of nematodes genera which represents 90% of the total
660 abundance in each sampling site Moinho (MOIRn), Navigator (NAVRn) and Tróia (TROIARn),
661 Replicates, n=1,2,3

662

663 **Figure 6.** Principal component analysis, based on Bray-Curtis Distances, according to nematode density at
664 Genus-level in each "sites" Moinho, Navigator and Tróia. PC1 = 40.9 % and PC2 =25 %. The vectors
665 represent the genera highest variability of the nematode assemblages

666

667 **Figure 7.** Constrained redundancy analysis displaying contributions of environmental factors to (**A**)
668 bacterial composition (RDA1= 66.9% and RDA2 = 14.7%) and (**B**) nematode assemblages (RDA1= 36.5%
669 and RDA2 = 24.5%)

670

671 **Tables**

672

673 **Table 1.** One-way PERMANOVA test, Beta-diversity of bacterial communities, between "Sites" (3 level
674 fixed) for all variables analysed, ($p \leq 0.05$), n=3

	Degree of freedom	*Sum squares*	*Mean square*	*Pseudo-F*	*P(perm)*	*Unique perms*	*P(MC)*
Bacterial abundance	2	1108.3	554.13	1.7106	0.007	280	**0.0983**
	6	1943.7	323.95				
	8	3052					

675

676 **Table 2.** Mean density ± standard error (SE) of the nematode genera (number of individuals per 10 cm^{-2}),
677 at each sampling site (Moinho, Navigator and Tróia). Trophic group (TG) and *c–p* value of each genera
678 included. Only the most abundant genera are included in this table

679

Genera	*TG*	*cp-value*	*Moinho*	*Navigator*	*Tróia*
Metachromadora	2B	2	7557.7 ± 803	412.8 ± 127	1872.5 ± 249
Terschellingia	1A	3	1868.2 ± 777	1413.8 ± 844	613.6 ± 111
Sabatieria	1B	2	1146.1 ± 653	256.2 ± 40	285.4 ± 50
Axonolaimus	1B	2	733.8 ± 294	12.5 ± 6	68.4 ± 11
Sphaerolaimus	2B	3	730.9 ± 410	45.7 ± 6	42.1 ± 4
Ptycholaimellus	2A	3	371.4 ± 113	19 ± 7	286.2 ± 212
Anoplostoma	1B	2	204.7 ± 94	131.5 ± 44	95.6 ± 56
Daptonema	1B	2	169.7 ± 68	23.8 ± 11	16.3 ± 12
Daptonema sp1	1B	2	149.1 ± 115	28.5 ± 9	59 ± 34
Spilophorella	2A	2	99.4 ± 77	0	7.9 ± 6
Microlaimus	2A	2	64.1 ± 49	52 ± 40	29.7 ± 23

Comesoma	1B	2	49.7 ± 38	3.2 ± 2	0
Dichromadora	2A	2	49.7 ± 38	7.9 ± 3	49.1 ± 38
Oncholaimellus	2B	3	49.7 ± 38	27.8 ± 21	0
Praeacanthonchus	2A	4	49.7 ± 38	3.3 ± 2	24.3 ± 10
Anticoma	1A	2	35.1 ± 27	0	0
Cyatholaimus	2A	2	35.1 ± 27	27.1 ± 11	0
Prochromadorella	2A	2	35.1 ± 27	0	0
Viscosia	2B	3	35.1 ± 27	34.9 ± 13	73.4 ± 47
Total			13466.8 ± 3745	2706.3 ± 1304	3842.8 ± 1036

680

Figures

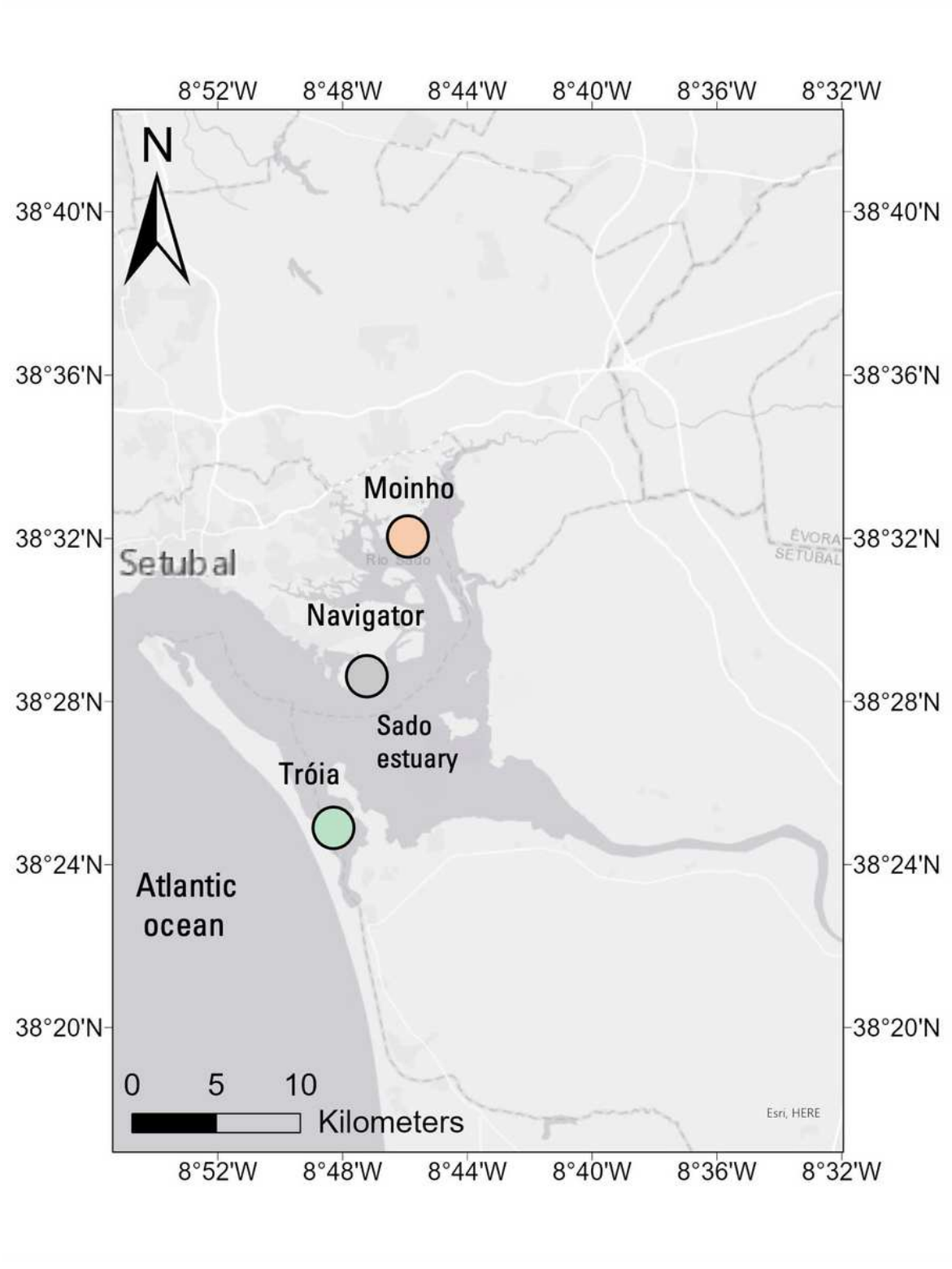

Figure 1

Sado estuary located at Southwest of Portugal (38° 31' 14" N, 8° 53' 32" W). The selected sampling sites: Navigator (38.487033, -8.795686) (grey circle), highly industrialized area; Moinho (38.528101, -8.802995)

(orange circle) with high organic inputs and Tróia (38.417317, -8.816433) (green circle) with coarser sediment. Moinho and Tróia are situated in a protected area

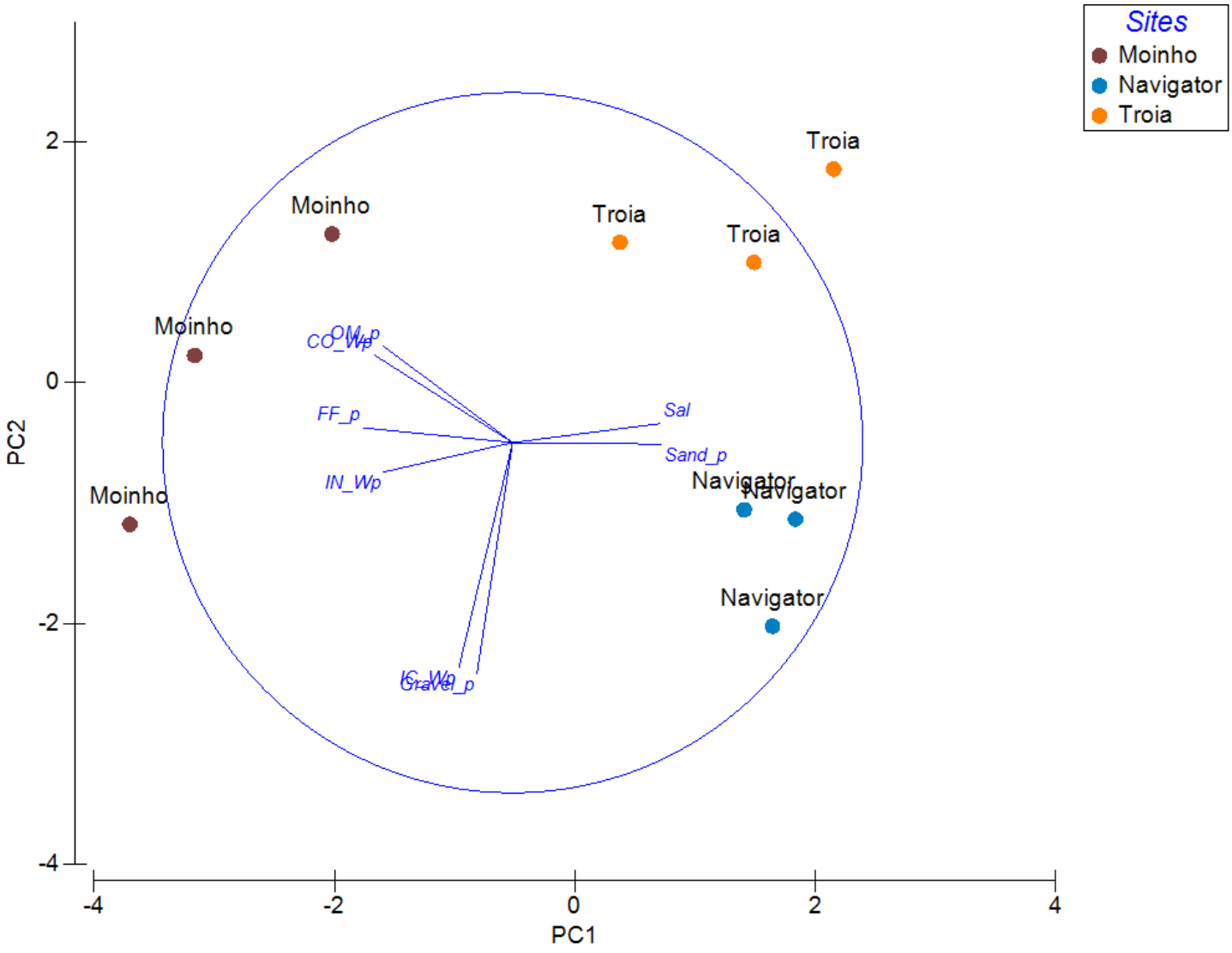

Figure 2

Principal component analysis, based on Bray-Curtis Distances, according to environmental variables measured (OM, gravel, sand, FF, IN, IC CO and salinity) at each sampling site: Navigator, Moinho and Troia, PC1 59.2%; PC2 31.4%

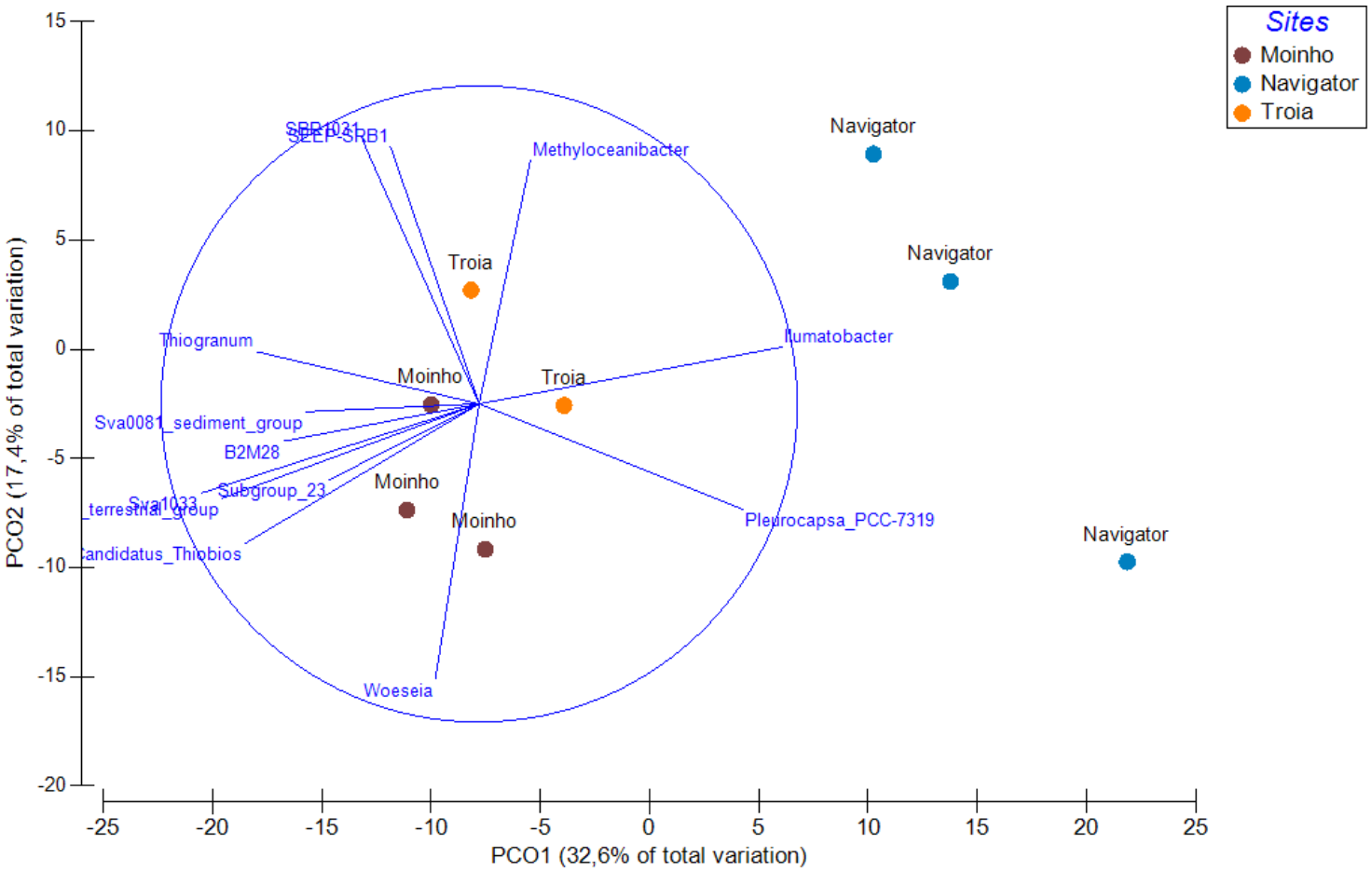

Figure 3

Principal component analysis, based on Bray-Curtis Distances, according to OTUs relative abundance of genera level obtained in each “site”. PC1 = 32.6 % and PC2 =17.4 %. The vectors are the most representative genera of the variability observed in bacterial communities

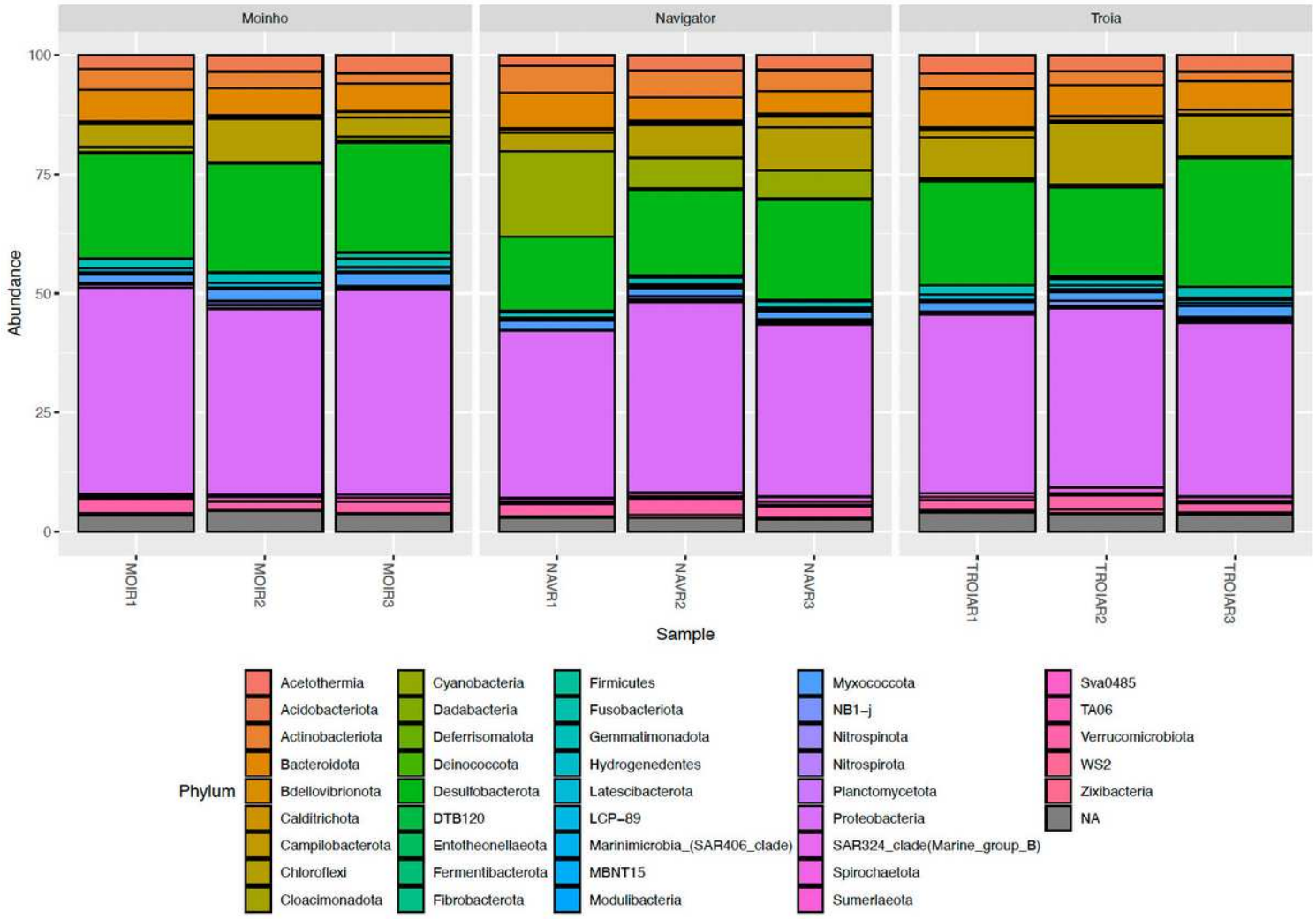

Figure 4

Bar plot displays the relative abundance of OTUs (%), based Phylum-level, that represents more than 90% of the total abundance in each site Moinho (MOI); Navigator (NAV) and Tróia (TROIA), n=3, the relative frequencies smaller than 1% collapsed into the N/A category

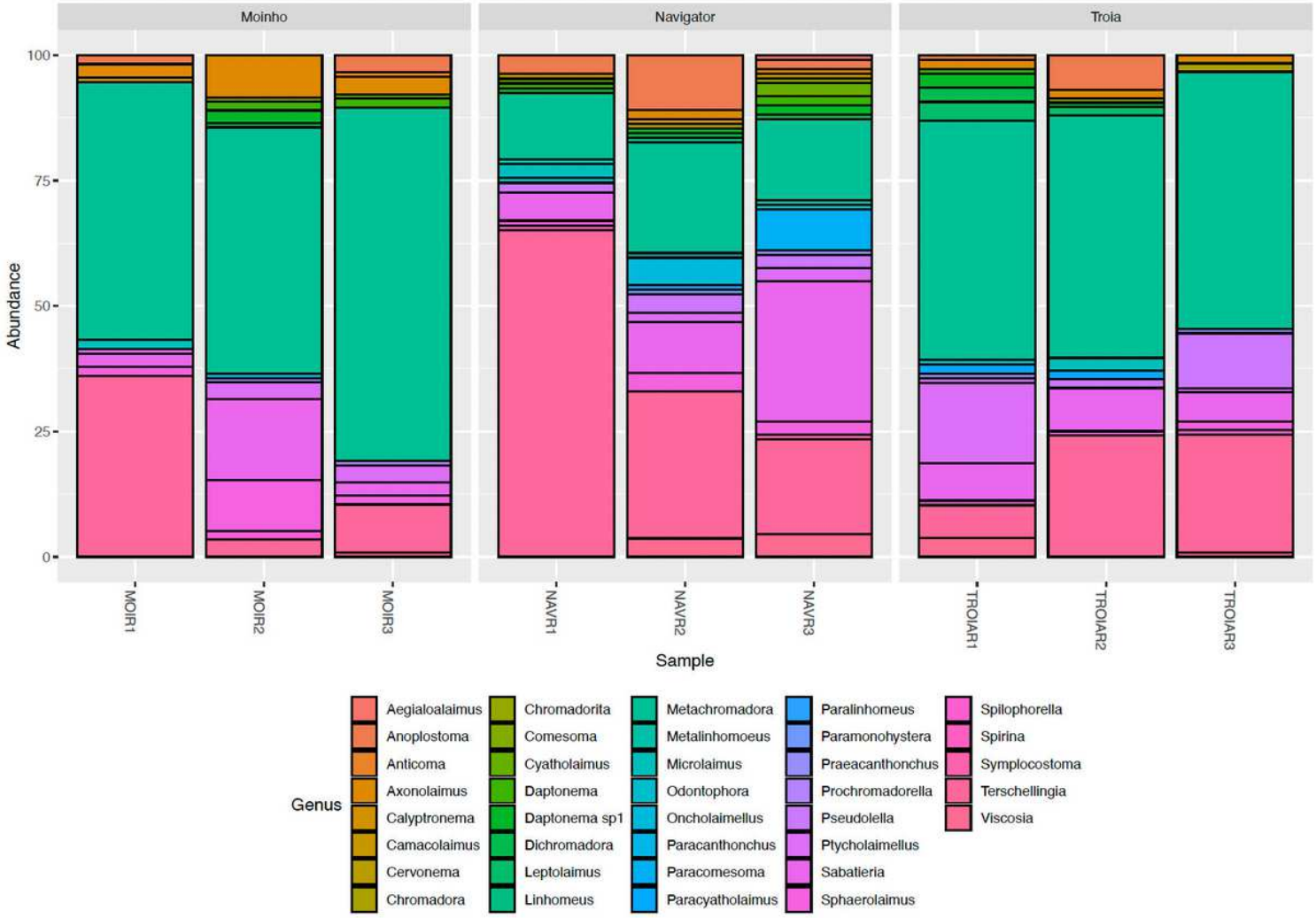

Figure 5

Bar plot displays the highest density of nematodes genera which represents 90% of the total abundance in each sampling site Moinho (MOIRn), Navigator (NAVRn) and Tróia (TROIARn), Replicates, n=1,2,3

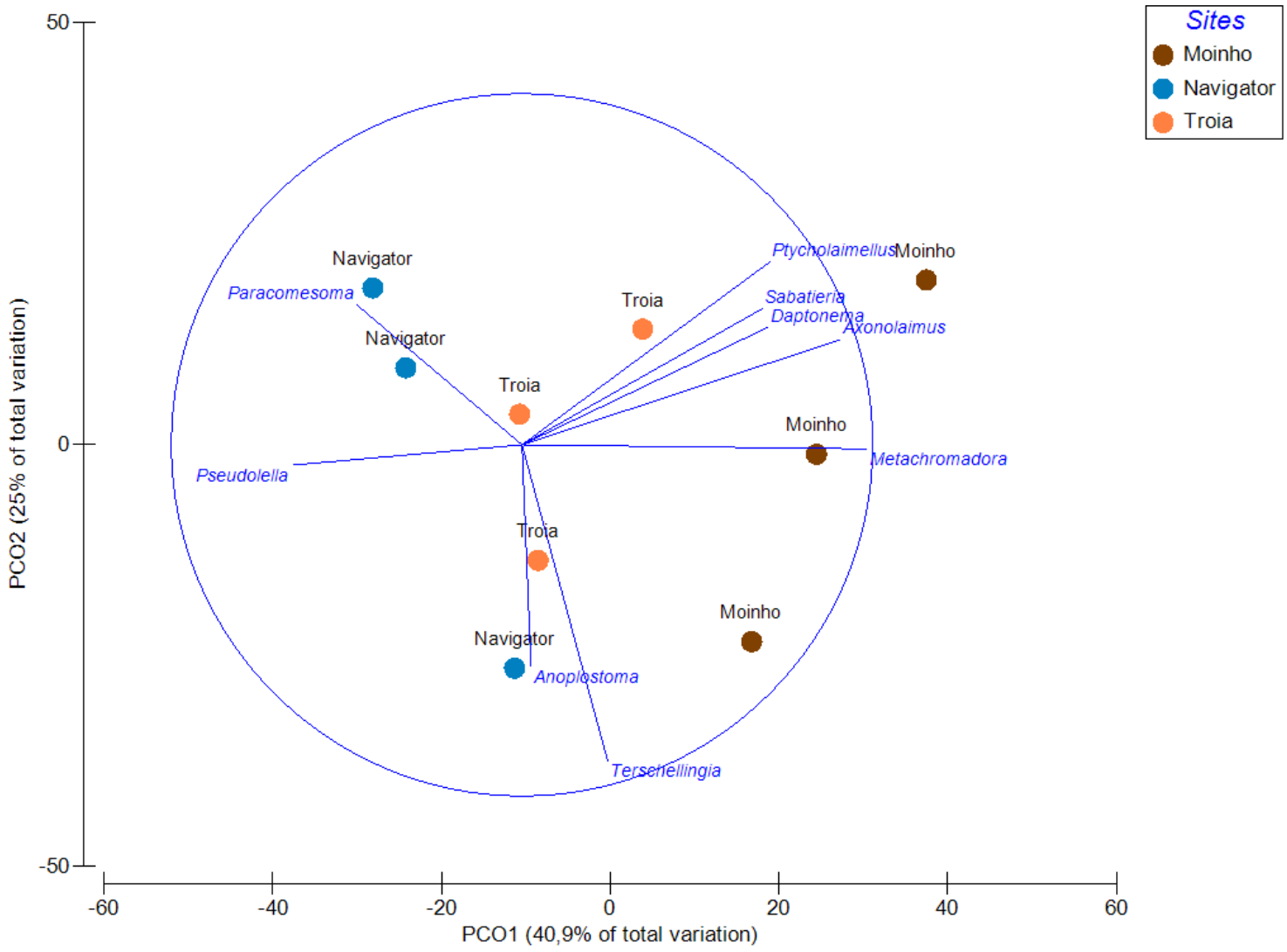

Figure 6

Principal component analysis, based on Bray-Curtis Distances, according to nematode density at Genus-level in each “sites” Moinho, Navigator and Tróia. PC1 = 40.9 % and PC2 =25 %. The vectors represent the genera highest variability of the nematode assemblages

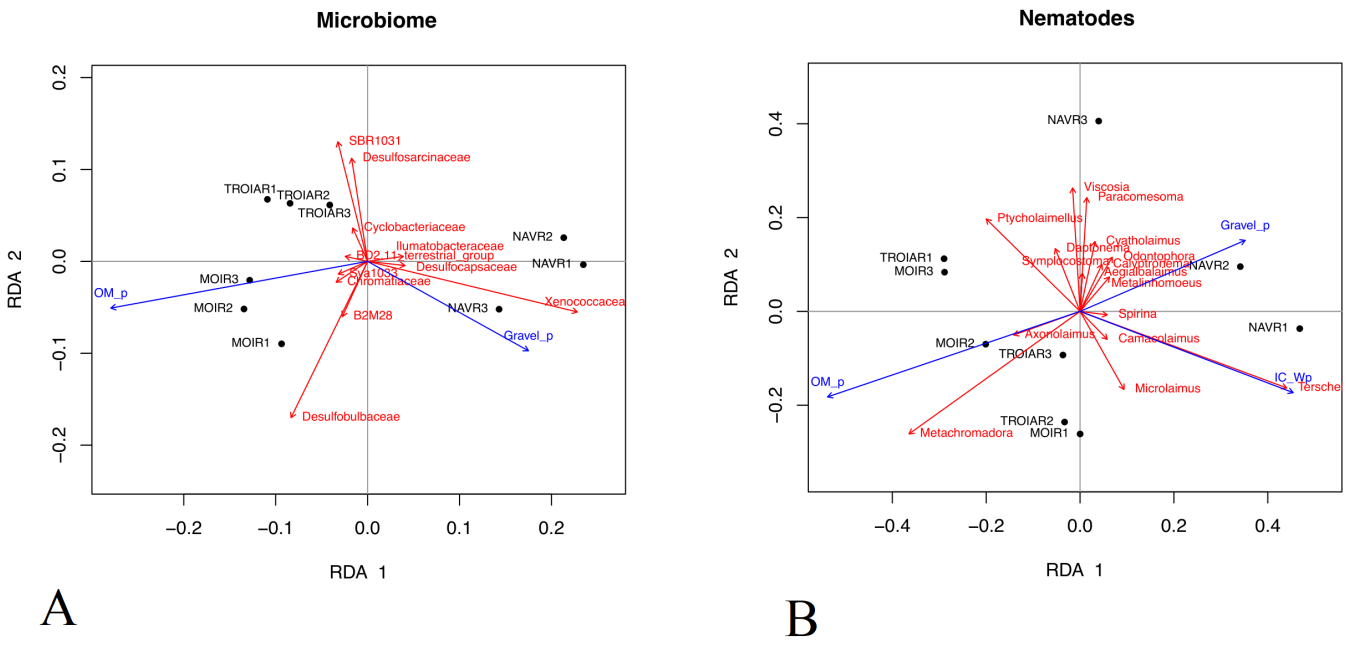

Figure 7

Constrained redundancy analysis displaying contributions of environmental factors to (A) bacterial composition (RDA1= 66.9% and RDA2 = 14.7%) and (B) nematode assemblages (RDA1= 36.5% and RDA2 = 24.5%)

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryTablesandFigures.docx