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# Distribution patterns of benthic bacteria and nematode communities in estuarine sediments

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ABSTRACT

Benthic organisms are crucial in the regulation of the ecosystem functions. The interactions between benthic nematodes and sediment bacteria across divergent environmental conditions are poorly understood. The main goal of this study was to understand the spatial distribution patterns and diversity of benthic bacterial communities and nematode assemblages of the intertidal sediments in three sampling sites (Navigator, Tróia and Moinho) along Sado Estuary (SW, Portugal). Bacterial communities were described using a 16S metagenomic approach, while nematode assemblages were characterized using morphological identification. Overall, bacterial and nematode communities presented significant diversity between sites ($p < 0.05$), which is primarily related with the environmental variables (*e.g.*, organic matter and percentage of gravel). The spatial distribution of bacterial communities was in accordance with the ecological conditions of three selected sites at a larger scale than nematode assemblages. Previously described as good ecological indicators, nematode assemblages were separated at sampling site level, suggesting that their response is driven by within site specific factors at a smaller scale. Hence, the present study set a fundamental ground for future research on functional interactions between bacteria and nematodes.

## 1. Introduction

Estuarine and coastal benthic ecosystems represent one of the major sources of essential services for human well-being (Bonaglia et al., 2014; Schratzberger et al., 2018). 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 higher trophic levels (Schratzberger et al., 2018). These functions are mediated by intra and interspecific interactions between organisms that support the functional integrity of the benthic ecosystems (Schratzberger et al., 2020).

Benthic nematodes are the most abundant taxon of metazoan meiofauna, representing 50–90% of total meiofauna abundance (Semprucci et al., 2014) and are considered an important tool to assess the effects of natural and anthropogenic disturbances in marine and estuarine sediments (Ridall et al., 2021). These organisms also play important roles in several ecosystem processes, being involved in complex relationships with microbial communities (Bonaglia et al., 2014; Nascimento et al., 2012; Derycke et al., 2016). The trophic composition of the nematode assemblages has been characterized by the morphological diversity of the buccal cavity providing feeding preferences or morphologic restrictions by ingesting certain type of food (e.g., bacteria or detritus). Under adverse environmental conditions, these assemblages can present a high trophic plasticity adopting generalist feeding behaviour (Nascimento et al., 2012; Derycke et al., 2016). Furthermore, nematode activities related with bioturbation, extracellular polymeric substances (EPS) production and grazing have been proved to be important contributors to stimulate the bacterial development and

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growth (Moens et al., 2005; De Mesel et al., 2006; D'Hondt et al., 2018). Nematodes are thus important mediators of energy transfer to higher trophic levels (Moens et al., 2005; De Mesel et al., 2006; D'Hondt et al., 2018; Vafeiadou et al., 2013), while sediment microbes are the primary facilitators of biogeochemical processes, such as carbon remineralization and sulphate reduction (Hargrave et al., 2008; Wang et al., 2020). A strong interconnection between nematode-microbe communities is well recognized, the presence of nematodes enhances bacterial metabolic activities, while bacteria provide physiological adaptations to nematodes under hypoxic and anoxic conditions (Bayer et al., 2009; Nascimento et al., 2012; Broman et al., 2020).

Assessing ecosystem conditions become one of the major concerns over the past two decades. Majority of the studies have been focused on the analysis of a single domain distribution patterns and relate with ecosystem environmental parameters (Materatski et al., 2015; Branco et al., 2018; Bulseco et al., 2020). However, such approach does not consider the interaction between organisms belonging to different domains, thereby limiting the assessment of the functional component of the ecosystem. Besides the existence of the above-mentioned associations between bacteria and nematodes, it is still 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 16S metagenomics to analyse the sediment bacterial 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 bacterial and nematode communities of the intertidal sediments in Sado Estuary in Portugal. The diversity patterns were investigated using: *i*) a 16S metagenomic approach for bacterial 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 of both communities will follow a close pattern, both responding to the environmental conditions of the sampling sites in Sado's Estuary.

## 2. Material and methods

### 2.1. 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 (Bettencourt et al., 2004) (Fig. 1). The intertidal areas comprise approximately 78 $km^2$, of which 30% are salt marshes and intertidal flats (Caeiro et al., 2005). 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. The salinity gradient ranges between 0.75 at upstream to 35.34 at downstream (Sroczyńska et al., 2021), and it is influenced by the Sado's river flow (annual mean of 40$m^3s^{-1}$) changing with seasonal and inter-annual conditions and temperature can range from 10 to 26 °C (Bettencourt et al., 2004).

The sampling sites were selected based on the expected differences in environmental conditions of the sediments according to water hydrodynamics within the Estuary (high/low water residence time), salinity gradient and the type of neighbouring anthropogenic activities (Caeiro et al., 2005; Kennedy et al., 2005; Sroczyńska et al., 2021). Based on above-mentioned criteria three "Sampling Sites" were selected (Fig. 1): (1) Navigator Site located in the proximity of industrial area, dominated by fine sand, clay and high organic contents (Caeiro et al., 2005); (2) Moinho Site is located within the borders of the Sado's Nature Reserve, affected by the surrounding aquaculture activities with the predominance of clay-fine sediments (Kennedy et al., 2005); (3) Tróia Site is located close to the Estuary mouth, is directly exposed to the main estuary channel, with high water exchange rate and high proportion of sand (Sroczyńska et al., 2021).

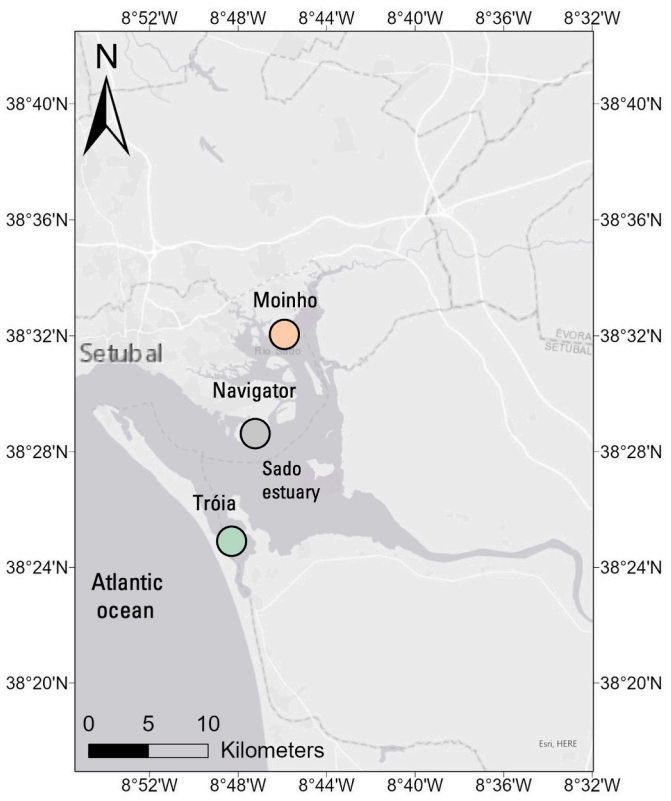


**Fig. 1.** Sado estuary located at Southwest of Portugal (38° 31′ 14″ N, 8° 53′ 32″ W). The selected sampling sites: Navigator (38.487033, −8.795686) (grey circle), highly industrialized area; Moinho (38.528101, −8.802995) (orange circle) with high organic inputs and Tróia (38.417317, −8.816433) (green circle) with coarser sediment. Moinho and Tróia are situated in a protected area. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Samples were collected between January and February of 2019 at each sampling site during neap low tide. At each site, three sediment samples were randomly collected for community analysis (nematode, n = 3; bacteria, n = 3) and for sediment physical-chemical analysis (n = 3).

### 2.2. Sediment physicochemical processing

The characterization of sediment samples included the analyses of total organic matter (OM) (%), granulometry, and elemental analysis (C and N), according to Costa et al. (2011). The sediment OM content (derived from the total combustible C content) was determined from the organic loss-on-ignition after burning samples at 400 °C for 3h. Gravel (>2 mm), sand (2–0.063 mm) and fine fraction (FF) (<0.063 mm) were determined by hydraulic sieving following disaggregation with pyrophosphate (Costa et al., 2011). For elemental analyses, each sediment sample was first dried (60 °C) to constant weight and subsequently grinded on a planetary micro mill Pulverisette 7 Classic Line from Fritsch. About 1.5 - 2.3 mg of grinded and combusted samples were placed in tin capsules (3.2 × 4 mm) and run in a TruSpec® Micro CHNS elemental analyser (Version 2.72) from LECO, for the simultaneous analysis of total C (TC) and N (TN), as described in (Teixeira et al., 2020). The independent infrared detectors detect the C content and the thermal conductivity detection system, the N content. The results are expressed as weight percentage (wt.%). The relative precision calculated from repeated measurement of samples and standards was 0.05%. In each sampling site, the salinity (SAL) of the sediment interstitial water was measured *in situ* using a VWR pHenomenal ® MU 600 H.

### 2.3. Sample processing of benthic communities

#### 2.3.1. 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 (⌀ 30 mm), snap-freezed in dry ice and transported into the lab, where were stored at −80 °C until DNA extraction. Total DNA extraction from 0.25 g of the sediment (surface between 0 and 3 cm) was conducted under sterile conditions, using the DNeasy® PowerSoil® kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. For cell lysis, samples were homogenized in a Precellys 24 Tissue Homogenizer (Bertin Instruments) for a total of 6 min under the program 2 × 20s 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) (Caporaso et al.,2012). A total of 9 samples were selected and sent for sequencing on Illumina MiSeq 2x 250bp (Illumina, Inc. San Diego, CA, USA) for INVIEW Microbiome at EUROFINS Genomics (Cologne, Germany). The protocol for preparation of the 16S rRNA gene library is detailed in 16S Metagenomic Sequencing Library Preparation Reference guide Part#15044223 Rev.B.

#### 2.3.2. Bioinformatics analyses and data availability

Raw Illumina data was demultiplexed and quality-filtered using the defaults parameters of QIIME2 (Bolyen et al., 2019). Single-end read data were denoised using DADA2 plugin (Callahan et al., 2016), 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 (Quast et al., 2013). Representative sequences were assigned taxonomy using a trained Naïve Bayes classifier (SILVA 138) (Bokulich et al., 2021) for V3-V4 hyper variable region from 16S rRNA. The resulting OTUs table was filtered to keep only features with a total abundance over 10. OTUs classified as chloroplast, mitochondria, eukaryote, archaea, and unassigned were also removed. Filtered OTUs table was rarefied at 14000 sequences per sample, the lowest sequencing depth (Appendix A, Fig. A1). 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).

#### 2.3.3. Nematode assemblages

Nematode samples were collected 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 $cm^{-3}$ (Heip et al., 1985). 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 (Vincx, 1996). Based on morphological characters, each nematode was identified until genus level (Olympus BX50 light microscope and cell software D Olympus, Japan). Taxonomic identification until genera, that is considered a level with good resolution in communities' assessment (Warwick et al., 1998) and was made using pictorial keys (Warwick et al., 1998; Platt and Warwick, 1983), and online identification keys/literature available in the Nemys database (Bezerra et al., 2021).

### 2.4. Statistical analyses

The statistical analyses of the nematode assemblages and environmental data was performed using the PRIMER v6 software package (Clarke &Warwick, 2001) with permutational analysis of variance (PERMANOVA) add-on package (Anderson et al., 2008). Statistical analyses of 16S rRNA metagenomics was performed in Quantitative Insights into Microbial Ecology (QIIME2, version 2020.8) (Bolyen et al.,2019) and *phyloseq* R package (McMurdie and Holmes, 2013).

#### 2.4.1. Environmental factors

To explore the multidimensional patterns of the environmental data, environmental matrix was analysed using Principal Component Analysis (PCA). The resemblance matrix was based on Euclidean distances and checked for uniform distribution, when necessary, the data was log (X+1) transformed and normalized (subtracting the mean and dividing by the standard deviation, for each variable) (Clarke &Warwick, 2001). The high correlated variables were selected and removed from the analysis.

#### 2.4.2. Bacterial and nematode communities

Bacterial communities α- and β-diversity indexes were calculated with q2-diversity plugin. For α-diversity analysis, several metrics were determined: Observed OTUs, Chao1, Shannon, Simpson, and Pielou's Evenness. To detect significant differences of α-diversity indices between sites, Kruskal-Wallis tests were performed, $p < 0.05$. To detect significant differences of β-diversity index was performed one-way PERMANOVA, $p < 0.05$.

To assess the diversity of nematode assemblages, Margalef's richness Index (d) (Margalef, 1958) and Shannon Wiener diversity ($H_0$) (Shannon and Weaver, 1963) were determined. To evaluate the trophic composition, each nematode genus was assigned to one of the four feeding groups based on mouth morphology, as follows: selective (1A) and non-selective (1B) deposit feeders, epigrowth feeders (2A) and omnivores/predators (2B) (Wieser, 1956). Based on the above-mentioned feeding-type classification, the reciprocal trophic diversity index ($ITD^{-1}$) was calculated to ascertain higher trophic diversity (Heip et al., 1985). The Maturity index (MI) was utilised as a life strategy measure, in which nematode genera were assigned to a colonizer/persister scale (*c-p* scale) varying between 2 (colonizers) and 5 (persisters) (Bongers et al., 1990; Bongers et al., 1991). One-way PERMANOVA analysis was performed to detect significant differences between nematode assemblages from each site, using the following design: fixed factor "Site" with 3 levels "Moinho"; "Navigator"; "Tróia", applying Bray-Curtis similarity matrix (Clarke &Warwick, 2001) with the significant level, $p < 0.05$. The same statistical test was performed for all diversity and functional descriptors to detect significant differences ($p < 0.05$) in the composition of the assemblages between "Sites". Data dispersions were inspected with PERMDISP and nematode density data were square root transformed.

Within both communities, the relative contribution of each taxon to the (dis)similarities between sites was calculated using the Bray Curtis method, SIMPER two-way crossover similarity percentage analysis (100% cut-off percentage).

#### 2.4.3. Environmental factors influencing the both communities

Redundancy Analysis (RDA) was conducted to test linear combinations of the environmental variables that best explain the variation of the bacteria and nematode communities' patterns. The response dataset consisted of Hellinger-transformed relative bacteria (observed OTUs with taxonomy assignment, corresponding to the 20 most abundant taxa) matrix, nematode genera abundance matrix (Legendre et al., 2001) 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", "SAL", "Sand", "TC" and "TN". "TN" was removed from the analysis due to high (>95%) correlation with" TC". All the variables were transformed using arcsine square root transformation, except for "TC" and salinity that was log10 transformed. A forward selection

procedure, using function "ordiR2step()" was utilised to select only significant variables ($p < 0.05$). RDA analysis was performed in R (Legendre et al., 2001) using "vegan" and "BiodiversityR" packages (Kindt and Coe, 2005). To test the correlation between both ordinations (bacteria and nematodes) it was performed Procrustean test. Procrustean test measures the degree of concordance between two or more datasets having different characteristics and if statistically significant, two datasets reflect in the same way the processes that determine their association (Peres-Neto and Jackson, 2001).

## 3. Results

### 3.1. Environmental variables

The environmental variables measured in sediment revealed three distinct sampling sites (Appendix B, Table B1). Moinho sediment was predominantly characterized by the highest mean values (%) of Gravel (2.27 ± 0.61), FF (80.9 ± 2.2), OM (11.1 ± 0.3), TC (1.46 ± 0.02) and TN (0.16 ± 0.002), while sediments from Tróia and Navigator presented the highest values of Sand (79.0 ± 7.2 and 78.6 ± 2.1, respectively) and SAL (35.2 ± 0.5 and 34.7 ± 0.13, respectively) (Appendix B, Table B1). These results were supported with a clear separation in the PCA (Principal Component Analysis) (PC1: 73.6% and PC2 22%) (Fig. 2). Moinho site was associated with high sediment OM content, FF and total TN, while Navigator and Tróia sites were associated with high SAL and % Gravel, respectively.

### 3.2. Sequencing statistics, diversity, and richness estimations

Illumina sequencing of the 9 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, 14000 reads were considered for further analysis after rarefaction (Appendix A, Fig. A1).

The estimated bacterial richness (Chao1) ranged between 608.2 ± 46.1 at Moinho and 670.3 ± 33.7 at Navigator (Table 1). While the diversity and evenness were similar between sites, no significant differences were obtained ($p = 0.670$) in accordance with Kruskal-Wallis test (Appendix B, Table B2). In terms of β-diversity, significant differences in bacterial composition (PERMANOVA, $p = 0.007$) were obtained between sampling sites (Table 2).

### 3.3. Bacterial composition, abundance across sites

Bacterial communities from all sites were composed by 53 phyla from which 18 phyla were the most representative, accounting for more than 90% of the taxa with more than 1% of the communities' total abundance (Fig. 3). The most relative abundant phyla were: *Pseudomonadota* (38–42%), *Desulfobacterota* (20–23%), *Chloroflexota* (5–9%), *Bacteroidota* (5–6%) and *Acidobacteriota* (2%). In all sampling sites the *Steroidobacterales, Desulfobacterales* and *Desulfobulbales* were the most relative abundant orders (Appendix A, Fig. A2), which were represented by high relative abundance of the families *Woeseiaceae, Desulfobulbaceae* and *Desulfosarcinaceae* and genera *Woeseia* and *Sva0081_sediment_group*. The bacterial community of the Moinho was distinguished from the others by the different relative abundance of common taxa and the genera *Sva1033* (3.6–3.7%), *B2M28* (1.9–2.8%) and *Candidatus Thiobios* (1.6–2.5%). While the Navigator community is distinguished from the others by the prevalence of the phylum *Cyanobacteria* which is exclusively represented by *Pleurocapsa_PCC-7319* genus (0–4%) (*Xenococcaceae*). Tróia's bacterial community was distinguished from the others by the different relative abundance of common taxa and the genera *SEEP-SRB1* (1.7–2.6%), *SBR1031* (1.8–3.7%) and *Sva1033* (2.7–3.6%). The SIMPER analysis showed that *Woeseia*, *Sva0081_sediment_group* and *Sva1033* (Similarity ≥68%) contributed the most for the similarity within the three sampling sites. Moreover, the great contributors for the major dissimilarities between sites were the genera *SEEP-SRB1* and *SBR1031* (Moinho vs Tróia, dissimilarity 29.94%), *Pleurocapsa_PCC-7319, Myxosarcina_GII* and *Cyanobacterium_CLg1* from the order *Cyanobacteriales* (Moinho vs Navigator and Navigator vs Tróia, dissimilarity 34–36%) (Appendix B, Table B3).

**Table 1**
Mean ± standard error (SE), n = 3 of Alpha diversity descriptors (Observed OTUs, Chao1, Shannon, Simpson and Pielou's Evenness) calculated for the bacterial communities from each sampling site: Moinho (MOI), Navigator (NAV), Tróia (TROIA).

| | Observed | Chao1 | Shannon | Simpson | Pielou's Evenness |
|---|---|---|---|---|---|
| MOI | 453.3 ± 30.5 | 608.2 ± 46.1 | 8.6 ± 0.1 | 0.9 ± 0.1 | 0.93 ± 0.0006 |
| NAV | 498.6 ± 30.1 | 670.3 ± 33.7 | 8.7 ± 0.1 | 0.9 ± 0.1 | 0.93 ± 0.004 |
| TROIA | 455.3 ± 43.3 | 616.1 ± 63 | 8.6 ± 0.1 | 0,9 ± 0.1 | 0.93 ± 0.001 |

### 3.4. Density and structural diversity of nematode assemblages

Overall, the nematode density varied between 2706.3 and 13466.9 individuals per 10 $cm^{-2}$ (Table 3). The nematode assemblages of Moinho site registered the mean density (13466.9 ± 1631.1 ind. 10 $cm^{-2}$), whilst the lowest mean density was obtained at Navigator sampling site (2706.4 ± 1092 ind. 10 $cm^{-2}$). PERMANOVA analysis for the nematode density revealed significant differences between "Sites", $p = 0.012$ (Appendix B, Table B4). The nematode assemblages of all sites were represented by the predominance of the orders Chromadorida, Monhysterida and Enoplida accounting for more than 80% of the total relative density. All assemblages were composed by the families Linhomoeidae, Desmodoridae and Comesomatidae. Nematode assemblages of Moinho are composed by 20 genera belonging to 13 families from which the genera *Metachromadora* (56%), *Terschellingia* (14%), *Sabatieria* (7%) and *Axonolaimus* (5%) account for 84% of the total relative density. The nematode assemblages collected at Navigator site were composed by 29 genera belonging to 14 families with genera *Terschellingia* (52%), *Metachromadora* (15%), *Sabatieria* (9%) and *Anoplostoma* (5%) accounting for 82% of the total relative density. The nematodes identified at Tróia sampling site were composed of 20 genera from 12 families with genera *Metachromadora* (49%), *Terschellingia* (16%), *Ptycholaimellus* (7%) and *Sabatieria* (7%) representing 80% of the assemblages collected (Fig. 4 and Appendix A, Fig. A3).

SIMPER analysis showed that *Metachromadora Terschellingia, Sabatieria* and *Axonolaimus* were the genera that most contributed for the similarity within each site, while *Metachromadora, Terschellingia* and *Axonolaimus* were the genera that most contributed for the dissimilarity between sites (Appendix B, Fig. B3). Although the assemblages of Moinho and Navigator were clearly separated, the nematode community patterns showed distinct spatial distribution comparing with bacterial communities.

### 3.5. Structural diversity, trophic composition and functional diversity

According to PERMANOVA analysis based on structural diversity descriptors (d, H′ ITD-1 and MI), significant differences were obtained between "Sites" ($p = 0.038$) for Margalef's richness index (d) (Appendix B, Table B4), whose highest diversity values were obtained at Navigator (Table 4). In addition to the high abundance of omnivores/predators (2B) at all sampling sites, particularly at Moinho (52% of total density), at Navigator selective (1A) and non-selective (1B) deposit feeders accounted for 63% of the Navigator assemblages. Tróia nematode assemblages were mainly comprised of omnivores/predators (2B: 45%) and selective deposit feeders (1A: 18%) (Table 3). PERMANOVA

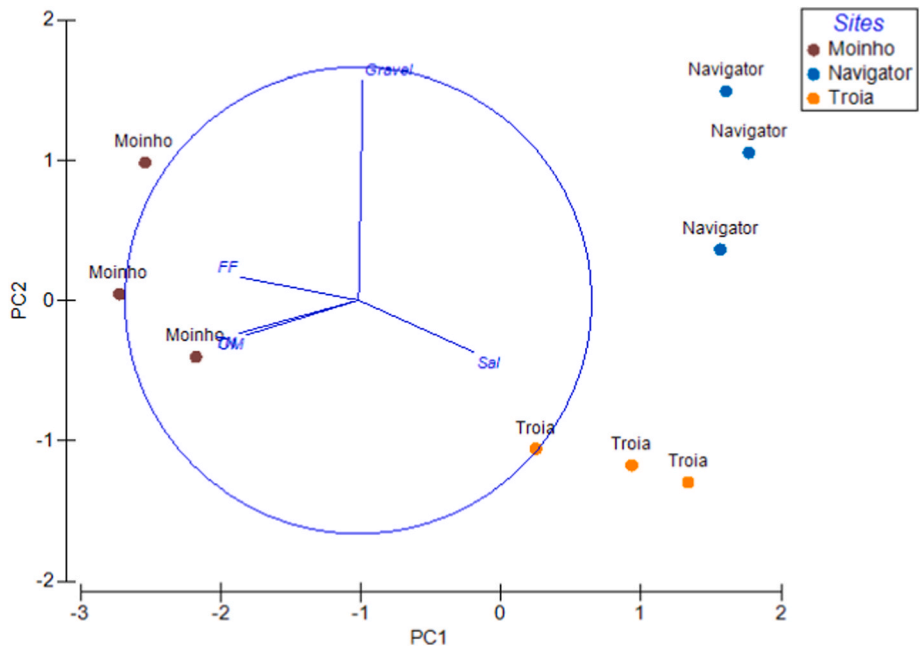


**Fig. 2.** PCA plot based on Euclidian distances, according to environmental variables measured (Organic Matter: OM, gravel, Fine Fraction: FF, Total Nitrogen: TN and Salinity: sal) at each sampling site: Navigator, Moinho and Tróia, PC1 73,6%; PC2 22%.

**Table 2**
One-way PERMANOVA test, Beta-diversity of bacterial communities, between "Sites" (3 level fixed) for all variables analysed, (p = 0.007), 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 | | | | | |

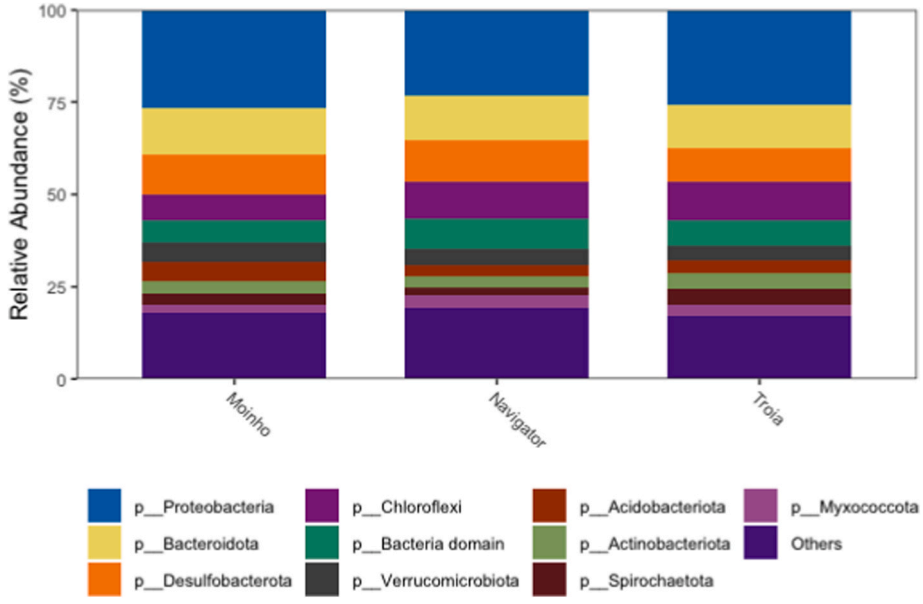


**Fig. 3.** Bar plot displays the relative abundance of OTUs (%). Representing the top 10 most abundant Phylum in each site Moinho; Navigator and Tróia, the other relative frequencies are collapsed into the Others category.

analysis of the nematode trophic composition data revealed significant differences between "Sites" (p = 0.0029) (Appendix B, Table B4).

The $ITD^{-1}$ values ranged from 1.7 to 3 and were very similar between assemblages for all sites. The MI varied from 2.1 to 2.7 and the highest mean value was obtained in Navigator assemblages. These results revealed the colonizer strategy (*c-p* value 2) dominated at Moinho (57%) and Tróia (42%). Navigator sampling site was PERMANOVA analysis of the functional diversity descriptors revealed no significance differences

**Table 3**
Mean density ± standard error (SE), n = 3, of the nematode genera (number of individuals per 10 $cm^{-2}$), at each sampling site (Moinho, Navigator and Tróia). Trophic group (TG) and *c–p* value of each genera included. Only the most abundant genera are included in this table.

| 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 |

**Table 4**
Mean ± standard error (SE), n = 3 of diversity descriptors of nematode assemblages ((S) genera, (d) Margalef, (H'(log.) Shannon (log based); ($ITD^{-1}$) reciprocal Index Trophic Diversity and (MI) Maturity Index). from each sampling site: Moinho (MOI), Navigator (NAV), Tróia (TROIA).

| Samples | S | d | H'(log.) | $ITD^{-1}$ | MI |
|---|---|---|---|---|---|
| MOI | 11.3 ± 0.9 | 1.1 ± 0.08 | 1.4 ± 0.1 | 2 ± 0.1 | 2.2 ± 0.06 |
| NAV | 17.6 ± 1.5 | 2.2 ± 0.3 | 1.9 ± 0.2 | 2.5 ± 0.2 | 2.4 ± 0.09 |
| TROIA | 13 ± 1.2 | 1.4 ± 0.1 | 1.6 ± 0.09 | 2.5 ± 0.15 | 2.3 ± 0.02 |

between "Sites" ($ITD^{-1}$, p = 0.246; MI, p = 0.064) (Appendix B, Table B4), highlighting the prevalence of similar trophic diversity and opportunistic strategies among nematodes inhabiting all three sites. dominated (46%) by the genera classified as *c–p* value 3.

### 3.6. Environmental variables influencing the spatial patterns of bacterial and nematodes assemblages

The RDA ordination on bacterial communities constrained by the environmental variables was highly significant (F = 5.44, p = 0.001, adjusted R2Ad = 0.53) (Fig. 5A). 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. 5A), 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 were strongly associated with NAVR3. *Desulfocapsaceae* and *Ilumatobacteraceae* families 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*, *Desulfosarcinaceae, Cyclobacteriaceae* as well as *BD2.11_terrestrial group* (Fig. 5A).

The RDA ordination on nematode's assemblages data constrained by the environmental variables was significant (F = 2.40 p = 0.023, adjusted R2Adj = 0.15), however after forward selection of the variables, solely OM emerged as a significant variable (p = 0.025), leaving only first RDA axis explaining 25% of the variability in the variance in nematode assemblages data (Fig. 5B). Contrary to bacterial communities, there is no obvious site separation, and it can be also observed a certain dispersal of all of the sites along both axes. Navigator sampling stations are separated from the remaining locations along the first axis. The only significant environmental variable emerged was OM that was highly associated to MOIR3 characterized the presence of *Metachromadora* genus. Genera associated to NAVR2 included *Odontophora* and *Calyptronema*. *Axonolaimus* was correlated with MOIR2 while *Metalinhomoeus* and *Spirina* were associated to TROIAR3. The presence of *Anoplostoma* was related to NAVR1. Moinho and Tróia were not separated at the RDA plot, but the separation of sites was rather exhibited at

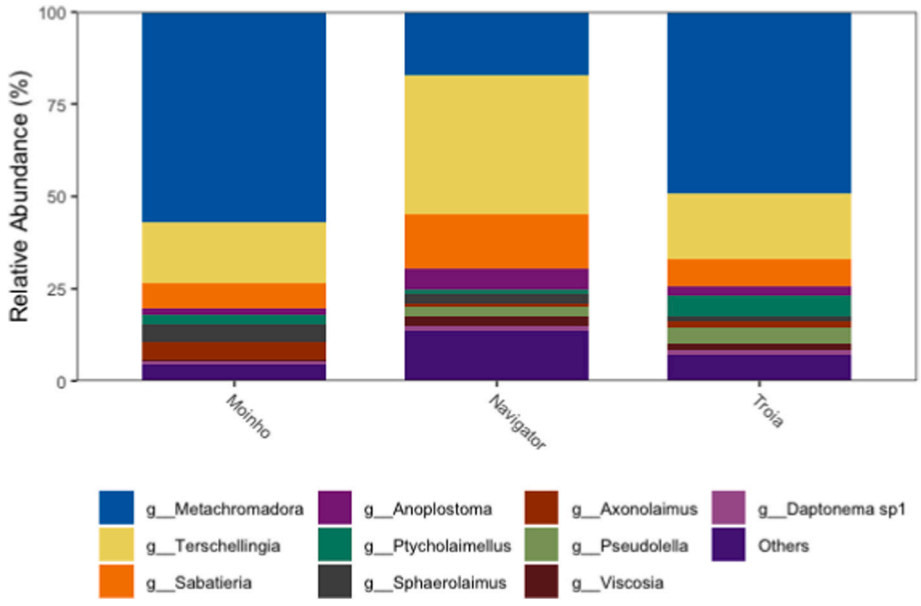


**Fig. 4.** Bar plot displays the relative density (%) of the top 10 most abundant of nematodes genera in each sampling site Moinho, Navigator and Tróia, the other relative frequencies are collapsed into the Others category.

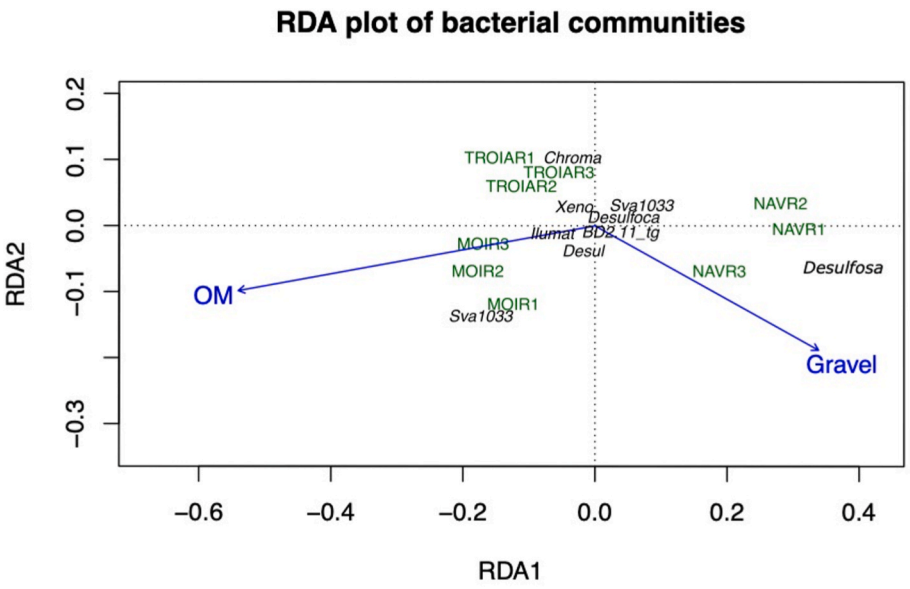


**Fig. 5a.** Constrained redundancy analysis displaying contributions of environmental factors to (**A**) bacterial composition (RDA1 = 56% and RDA2 = 8%). The species that are displayed in the graph have a goodness of fit higher than 0.4.

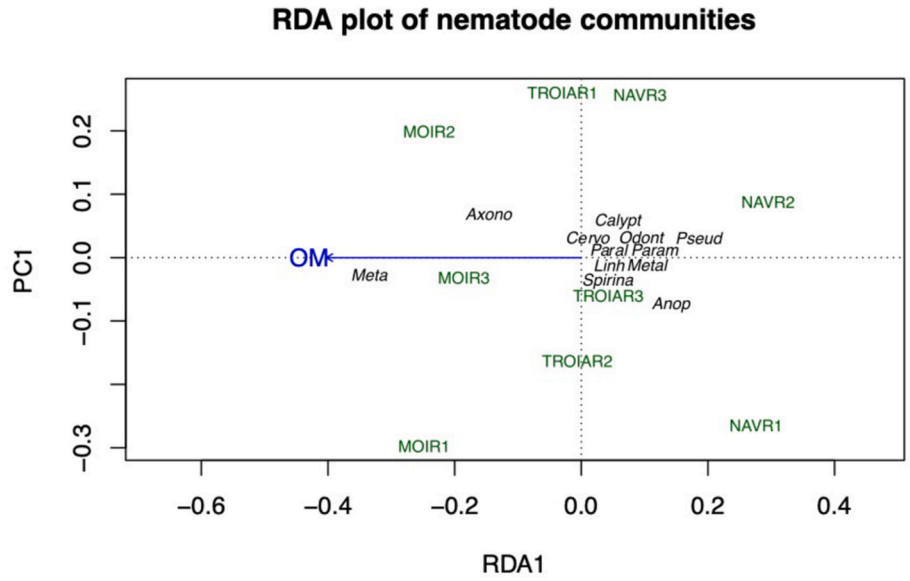


**Fig. 5b.** Constrained redundancy analysis displaying contributions of environmental factors to (**B**) nematode assemblages (RDA1 = 25.5% and RDA2 = 28.5%). The species that are displayed in the graph have a goodness of fit higher than 0.2.

the level of sampling stations, within each site than by the level of sites. The result of Procrustean test that analysed the correlation between both ordinations was not significant (Correlation in a symmetric Procrustes rotation: 0.6743, p = 0.142) indicating significantly different patterns in the ordination for nematode and bacterial communities.

## 4. Discussion

The high spectrum of environmental conditions registered in estuarine sediment are well known to capture a variety of adaptive responses in benthic communities (Sroczyńska et al., 2021). In this study, the spatial distributions of bacterial and nematode assemblages were studied at three different sites of Sado Estuary to evaluate the hypothesis that spatial distribution of both communities follow close patterns, responding congruently to the sediment conditions. Concerning environmental variables, the results showed a clear spatial distribution pattern undermining significant differences between sites for both communities. Still, we could not observe these patterns between bacterial communities and nematodes assemblages: bacterial communities of Navigator were separated from the other communities, while nematode assemblages of Moinho were set apart from the Navigator and Troia sites.

The influence of the environmental conditions on the spatial distribution patterns of the bacterial communities is well known (Jessen et al., 2017). *Desulfobacterota* was one of the most abundant phyla present at all sites, known to play important roles in most of the biogeochemical processes in the anoxic layer of estuarine sediments (e.g., anaerobic processes in S and C cycles) (Baker et al., 2015; Jessen et al., 2017; Raggi et al., 2020). 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 (Spring et al., 2015; Marshall et al., 2021). Symbiotic

organisms from the family *Rhodobacteraceae* (De Mesel et al., 2006) 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) (Ott et al., 2004; Bayer et al., 2009; Zimmermann et al., 2016). 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 OM (Raggi et al., 2020). Cyanobacteria phylum was responsible for separating bacterial communities of Navigator from the other sites, with the exclusive presence of the unicellular and pseudo-filamentous genus *Pleurocapsa* (Kolda et al., 2020). These organisms are regarded as ecological important groups in estuarine and coastal environments being primary producers and N/C fixators. Their increased growth highlights the presence of opportunistic species with the release of cyanotoxins (Kolda et al., 2020). The distribution patterns of bacterial communities at the Navigator site are strictly related with the high proportions of %Gravel and SAL, which is in accordance with Kolda et al. (2020), that showed the preference of cyanobacteria for a sandy gravel type of sediment.

At Sado Estuary, the density of nematode assemblages were high considering other Portuguese estuaries (SW coast of Europe) such as Mira and Mondego (Alves et al., 2013; Materatski et al., 2015). The Moinho sampling site registered the highest nematode density, which may be related with high OM content available at the bottom of the sediments (Moens et al., 2005; Adão et al., 2009). The predominance of sandy sediments in Navigator and Tróia sampling sites contributed to the low nematode density but a diversity increase, possibly related with the broader range of microhabitats available for nematodes in these sediments when compared to muddy ones (Steyaert et al., 2003). In all sampling sites, the dominant genera were *Metachromadora, Sabatieria, Axonolaimus* and *Terschellingia*, similar composition to mud-flat areas of Mondego estuary (Alves et al., 2013) and also to the previous study in Sado estuary (Sroczyńska et al., 2021). The spatial distribution patterns base on $ITD^{-1}$ and MI indexes were also similar to those verified in Mondego, Mira and Sado estuaries (Alves et al., 2009; Materatski et al., 2015; Sroczyńska et al., 2021a). However, the trophic composition of nematode assemblages showed different results from previous studies (i.e., usually dominated by non-selective deposit feeders (1B) and epistrate feeders (2A) (Alves et al., 2013; Branco et al., 2018). The omnivores/predators (2B) were abundant in all sampling sites, which are mainly grazers of microphytobenthos and bacteria (D'Hondt et al., 2018; Van der Heijden et al., 2019). These organisms are usually able to vary in their feeding mode in response to the food availability being difficult to draw a general trend in abundance of these feeding groups (De Mesel et al., 2006; Van der Heijden et al., 2019). Nematode assemblages sampled in Navigator showed a high percentage of non- and selective deposit feeders (1A and 1B) such as *Terschellingia* and *Sabatieria,* which are usually favoured by the depositional nature and hypoxic conditions of the sediment. These observations highlighted the possibility that the bacterial composition might be related to the feeding preferences of nematodes and to their affinity for cyanobacterial biofilms (Derycke et al., 2016; D'Hondt et al., 2018). Moreover, Vafeiadou et al. (2013) and Sahraean et al. (2017) also demonstrated by stable isotope analysis that *Terschellingia* can thrive under conditions that benefit the chemoautotrophic prokaryotic activity by using methane-derived carbon as energy source. Despite the high abundance and co-occurring of *Terschellingia* and *Cyanobacteria* at Navigator, it was not possible to draw conclusion about the type of nematode-bacteria interactions at this stage. Apart from the shared environmental preferences and other indirect relationships, further hypothesis-based studies are needed to better understand the potential interactions between co-occurring taxa.

The RDA analysis demonstrated that variables that contribute most to spatial distribution patterns for both nematode and bacterial communities, were OM and %Gravel. Besides bacterial-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 the majority of the variability occurred in bacterial community data. These observations were not reported by other studies, so far. Nematode assemblages are considered a good ecological indicator to specific sampling locations (Branco et al., 2018). In this study, the distribution patterns of this community may suggest that their responses are driven by site specific factors, acting at the small spatial scale.

## 5. Concluding remarks

Using a multivariate approach on two datasets delivered from metagenomic assessment (16S rRNA amplicon sequencing) of bacteria and morphological assessment of nematodes allowed us to analyse the spatial distributional patterns under different ecological sediment conditions. We conclude the spatial pattern of nematodes is driven by small-scale factors within each site, explained by the sediment OM content. However, the spatial pattern of bacteria is driven by factors acting on larger scale between sites, explained by the %Gravel and sediment OM content. The methodology applied was not sensitive enough to ascertain estuarine sediment bacteria-nematode interactions. However, the spatial patterns presented by both communities in this study set a fundamental ground for future research on functional interactions between bacteria and nematodes.

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## Code availability

Not applicable.

## Ethics declarations

Not applicable.

## Consent for publication

Not applicable.

## CRediT authorship contribution statement

**Soraia Vieira:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Formal analysis. **Kasia Sroczyńska:** Writing – review & editing, Methodology, Formal analysis. **Joana Neves:** Methodology. **Marta Martins:** Writing – review & editing, Methodology. **Maria Helena Costa:** Writing – review & editing. **Helena Adão:** Writing – review & editing, Resources, Project administration, Funding acquisition, Conceptualization. **Cláudia S.L. Vicente:** Writing – review & editing, Visualization, Supervision, Methodology, Investigation, Formal analysis, Conceptualization.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecss.2023.108448.

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