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BRIEF REPORT

REVISED Tagus River microbial profile through nanopore sequencing on samples gathered from Prainha do Braco de Prata, Lisbon

[version 3; peer review: 1 approved, 2 approved with reservations]

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Abstract

Background

Freshwater ecosystems play a vital role for hosting life, and their study can elucidate their dynamic state throughout time. However, there is not much knowledge about the microbial profiles and their relevance for the ecosystem balance is still unclear.

Methods

In this Brief Report three freshwater samples collected in the Tagus River north margin were analysed through 16S-targeted nanopore sequencing and by customized bioinformatics pipeline.

Results

Our results revealed a consensual microbial profile with Candidatus *Pelagibacter*, *Egibacter*, and *Ralstonia* as the most abundant genera. Additionally, through a literature review we found that the ecosystem services provided by these genera are mostly related to organic matter decomposition.

Open Peer Review

Approval Status ? ? ✓

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version 2 (revision) 05 Dec 2024		? view	? view
version 1 29 Jul 2024	? view	? view	

1. **Dave R. Clark**, University of Essex, Colchester,, UK
2. **Anyi Hu**, Chinese Academy of Sciences, Xiamen, China
3. **Sushanta Deb**, Washington State University, Pullman, USA

Any reports and responses or comments on the article can be found at the end of the article.

Conclusions

Despite the need for a more robust sampling and analyses, we conclude that there is potential to use microbial profile approaches to help define the relevant microbial biomarkers to clarify the ecosystem services in the Tagus River freshwater ecosystem.

Keywords
Tagus River, Freshwater microbiota, Nanopore sequencing, Microbiota, Lisbon

HE This article is included in the Horizon Europe gateway.

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REVISED **Amendments from Version 2**

In this version we extended the methods section to accommodate the comments made by Dr Sushanta Deb. We also provide a figure-style to show the most abundant taxa found in four taxonomic rabks. However, it is our perspective that the relevancy of the discussion should stay at lower taxonomic ranks and not at the highst taxonomic ranks as suggested. Still, we would like to see what is the perspective of Dr Sushanta on the matter.

In respect to the Dr Anyi Hu comments on the "I think it would be better to provide some environmental parameters, such as salinity etc., which can show the investigated area was under the influence of sea intrusion", we cannot provide such data because we did not collect them. So this fact constitute a limitation of our brief report.

Any further responses from the reviewers can be found at the end of the article

Introduction

A complete comprehension of the Tagus River ecosystem is lacking. Due to the transboundary relevance of the Tagus River, its comprehension is paramount. Moreover, there is also insufficient information about the present and future impact posed by challenges in water management (Sondermann & de Oliveira, 2022), by climate change (Sondermann & de Oliveira, 2022), and anthropogenic alterations (Fernandes *et al.*, 2020).

In terrestrial ecosystems most biomass comes from plants, where microbial biomass accounts for ~70% in the marine ecosystems (Bar-On *et al.*, 2018). As rivers work as flows of nutrients and water it is relevant to get a clearer picture on the role of their microbiomes and phenotypes (Reddington *et al.*, 2020; van Rossum *et al.*, 2015). Since microorganisms have short lifecycles and large population size so their organization and structure correspond to a swift adaptation to the shifting environmental changes (Cavan *et al.*, 2019; Collins *et al.*, 2014; Hellweger *et al.*, 2014). The global climate crisis is pressing the changes in marine ecosystems threatening the services they provide. This happens by major variations in the species present in marine habitats. To assess such fast shifts unified frameworks need to be developed that uses data from ecology, metabolism, and climate change (Baltar *et al.*, 2019). Without this knowledge, the ability to understand, evaluate, and forecast the future of marine communities, ecosystems, and their services is almost inefficient.

In this Brief Report we wanted to elucidate the microbial profile of a coastline location in the Tagus River estuary (Beato, Lisboa), and infer the potential causes and consequences of the presence of the observed taxa.

Methods

Samples collection

The environmental water samples were collected in Praínha de Braço de Prata (Figure 1). Three water samples were collected using three sterile plastic containers (5 L) for microbiota analysis. The containers were submerged at 0.5 meters and opened below surface to guarantee no air-borne microorganisms' collection.

Total DNA was extracted from 5 L of each sample collected from the Tagus River and sequentially filtered through 20 µm, 3 µm, 0.45 µm, and 0.22 µm filters. The 0.45 µm and 0.22 µm filters were combined into a single 5 mL PowerWater Bead Pro Tube for DNA extraction using the DNeasy PowerWater Kit (Qiagen) according to the manufacturer's instructions. The purity of the extracted DNA was evaluated by measuring absorbance at 260 nm with a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, USA). The DNA was stored at –20 °C until further processing.

Amplification of then 16S rDNA gene and sequencing library preparation

For the rDNA gene amplification, Long Amp hot start Taq 2× master mix (New England Biolabs, MA, USA) was used at

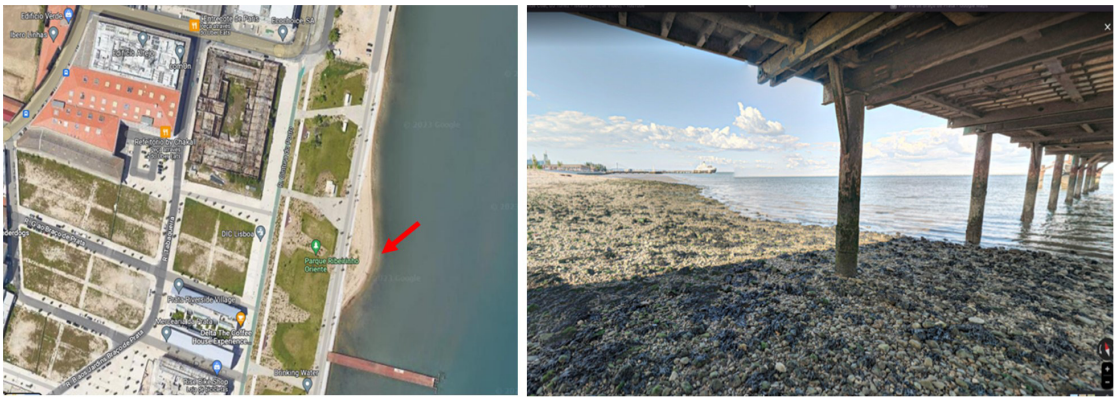

Figure 1. Sample collection site – 'Praínha de Braço de Prata'. Left: aerial view map of the site (red arrow). Right: photo of the site taken in May 2020 by Miguel Silva. Google Maps (n.d.) (View from Praínha de Braço de Prata). Retrieved in July 13, 2023, from https://www.google.com/maps/place/38°44'45.9%22N+9°05'47.2%22W/.

1X along with 50 ng/μL of genomic DNA from Tagus River water samples. To amplify the full-length 16S rDNA bacterial gene, 0.25 μM of the primer pair 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-CGGTTACCTTGTTACGACTT-3′) were used. Both PCRs were conducted on a Biometra UNO II, using the following conditions for 16S rDNA gene: 1 cycle of 94 °C for 1 min, 35 cycles of 94 °C for 20 s, 55 °C for 30 s, and 65 °C for 2 min, and a final extension of 65 °C for 5 min. Subsequently, amplification products were visualized via gel electrophoresis and purified using the Solid Phase Reversible Immobilization (SPRI) technique with magnetic beads (Stotchevoi *et al.,* 2020). The library was prepared from 200fmol input DNA from each sample using the Sequencing Native Barcoding Kit 24 V14 (SQK-NBD114.24) (Oxford Nanopore Technologies, Oxford, UK) in accordance with the manufacturer's protocol.

Sequencing run

Sequencing runs were performed using R10.4.1 flow cells on a GridION sequencing platform and sequencing data were acquired in real time using MinKNOW 18.08.2 software. Sequencing data was stored in fastq files, with each file representing a batch of 4000 reads.

Bioinformatics analysis

Sequencing data was derived from 16S amplicons, with the exclusion of low-quality reads with the following parameters: a minimum quality score of 10, a minimum read length of 1200 bps, and the removal of reads with ambiguous bases (N's). The remaining reads underwent size selection, retaining those with lengths between 1200 bps and 1700 bps, accomplished through *prinseq-lite* (Schmieder & Edwards, 2011).

For taxonomic classification, we employed a Lowest Common Ancestor (LCA) approach. Clean reads were classified with Kraken version 2.1.2, using a reference database that includes a comprehensive collection of high-quality, annotated 16S rRNA sequences. The database used was the RefSeq plus GenBank Archaea and Bacteria database, created in May 2023. The classification process assigns each read to the lowest common ancestor of all genomes known to contain the specific sequence patterns identified in the read. This approach ensures robust taxonomic assignment by resolving ambiguities in read classification. For post-classification, the data underwent rarefaction and was subjected to several analyses: i) alpha-diversity group significance analysis (Bolyen *et al.*, 2019); and ii) sample dissimilarity analysis - Principal Coordinates Analysis (PCoA): Applied for beta-diversity analysis based on the Bray-Curtis similarity index. Taxa abundance was determined, employing a genera prevalence cutoff of ≥ 0.01 (Bolyen *et al.*, 2019). The sequencing depth for rarefaction was standardized to the sample with the lowest read count, which is 339,909 reads. This ensures that all samples are compared on an equal footing, avoiding biases introduced by differences in sequencing depth. In the rarefaction process, reads were randomly subsampled to match the sequencing depth of the sample with the lowest readcount (339,909 reads). This step was implemented to standardize the dataset while preserving the relative abundances of taxa within each sample. Then, data were subjected to determination of taxa abundance (with a genera prevalence cutoff of ≥ 0.01) (Bolyen *et al.*, 2019) and Krona Plots (by hierarchical order of Operational Taxonomic Units frequency).

The validation of our bioinformatics approach was performed using the ZymoBIOMICS™ Microbial Community Standard. Specifically, we sequenced this mock community using 16S rRNA amplicon sequencing and analyzed the data with the same pipeline applied to our samples. The proportions of sequencing reads classified to each taxon closely matched the expected proportions in the mock community. This agreement between the observed and expected proportions confirms the accuracy of our methodology in reflecting community composition, thereby validating the reliability of our approach for the samples studied.

For post-classification, the data underwent rarefaction and was subjected to several analyses: i) alpha-diversity group significance analysis (Bolyen *et al.*, 2019); and ii) sample dissimilarity analysis - Principal Coordinates Analysis (PCoA): Applied for beta-diversity analysis based on the Bray-Curtis similarity index. Taxa abundance was determined, employing a genera prevalence cutoff of ≥ 0.01 (Bolyen *et al.*, 2019). The sequencing depth for rarefaction was standardized to the sample with the lowest read count, which is 339,909 reads. This ensures that all samples are compared on an equal footing, avoiding biases introduced by differences in sequencing depth. In the rarefaction process reads were randomly subsampled to match the sequencing depth of the sample with the lowest read count (339,909 reads). This step was implemented to standardize the dataset while preserving the relative abundances of taxa within each sample.

Results

Nanopore sequencing general output

The three samples showed similar quality parameters which gives confidence that the sample collection and the sequencing approach were appropriately performed (Table 1). Specifically, the mean read length is equal in the three analyses (1,570-1,574 bases), the same for the mean read quality (Q = 17.7-18.0), and the percentage of reads above the cutoffs. The sample 2 produced less reads (and bases) than the other two samples, but that did not hamper the quality of the output.

Prainha de Braço de Prata microbiota

The top main phylum, genus, and species observed in the three water samples are shown in Table 2.

In order to assess the Praínha do Braço de Prata microbiota we qualified and quantified the shared taxa between the three samples (Figure 2).

Table 1. Main nanopore sequencing quality parameters from the three environmental samples.

Quality feature (units)	Sample 1	Sample 2	Sample 3
Total number of reads (thousand)	>534	>339	>474
Total number of classified reads (%)	99.82	99.87	99.91
Total number of bases (million)	>841	>533	>746
Mean read length (bases)	1,574	1,570	1,571
Read length N50 (bases)	1,594	1,584	1,582
Mean read quality (Q score)*	17.5	18.0	18.0
Reads above quality 7 cutoff (%)	100.0	100.0	100.0
Reads above quality 10 cutoff (%)	100.0	100.0	100.0
Reads above quality 15 cutoff (%)	84.1	84.3	84.7

* Q score of 7 means a basecall accuracy of 80%, Q score of 10 means a basecall accuracy of 90%, and a Q score of 15 means an accuracy of 96.8%

Table 2. Three top phylum, genera, and species identified in the three samples with their respective relative abundance.

Sample	Rank	Taxa (relative abundance, %)
1	Phylum	*Pseudomonadota* (36), *Actinomycetota* (10), *Bacteroidota* (11)
	Family	*Flavobacteriaceae* (10), *Roseobacteraceae* (10), *Egibacteraceae* (7)
	Genus	*Egibacter* (7), *Tateyamaria* (6), *Ralstonia* (6)
	Species	*Egibacter rhizosphaerae* (7), *Tateyamaria omphalii* (6), *Ralstonia solanacearum* (6)
2	Phylum	*Pseudomonadota* (65), *Actinomycetota* (15), *Bacteroidota* (15)
	Family	*Burkholderiaceae* (14), *Roseobacteraceae* (14), *Flavobacteriaceae* (13)
	Genus	*Ralstonia* (14), Candidatus *Pelagibacter* (11), *Egibacter* (10)
	Species	*Ralstonia solanacearum* (14), Candidatus *Pelagibacter* sp. (11), *Egibacter rhizosphaerae* (10)
3	Phylum	*Pseudomonadota* (45), *Actinomycetota* (13), *Bacteroidota* (10)
	Family	*Pelagibacteraceae* (17), *Egibacteraceae* (10), *Flavobacteriaceae* (9)
	Genus	Candidatus *Pelagibacter* (17), *Egibacter* (10), *Ralstonia* (7)
	Species	Uncultivated bacterium from the SAR86 clade (16), *Ralstonia solanacearum* (10), *Egibacter rhizosphaerae* (7)

The microbial profiles differed between sample 1 and sample 3 at the phylum, genus, and species level (Shannon index; p-value < 0.05). The sample 1 and sample 2 showed similar alpha-diversities (Shannon index; p-value > 0.05). The samples showed incongruent dissimilarities microbial profiles amongst them. There were no differences amongst the three samples' microbial profiles at the genus level. At the species level, the microbial profile from sample 3 is different from sample 1 and 2, but sample's 2 and sample's 1 microbial profile are similar. Lastly, he phylum level, the microbial profile from sample 2 is different from sample 1 and 3, and sample's 1 shows a similar microbial profile than sample 3 (PERMANOVA $R^2 = 1$).

Ecosystem services

Based on the shared taxa found between the three samples we used the top three genus and species to assess in PubMed database studies that inform the potential ecosystem services these taxa might be performing in the Tagus River. In total it was identified 17 articles related to *Pelagibacter* gen. and

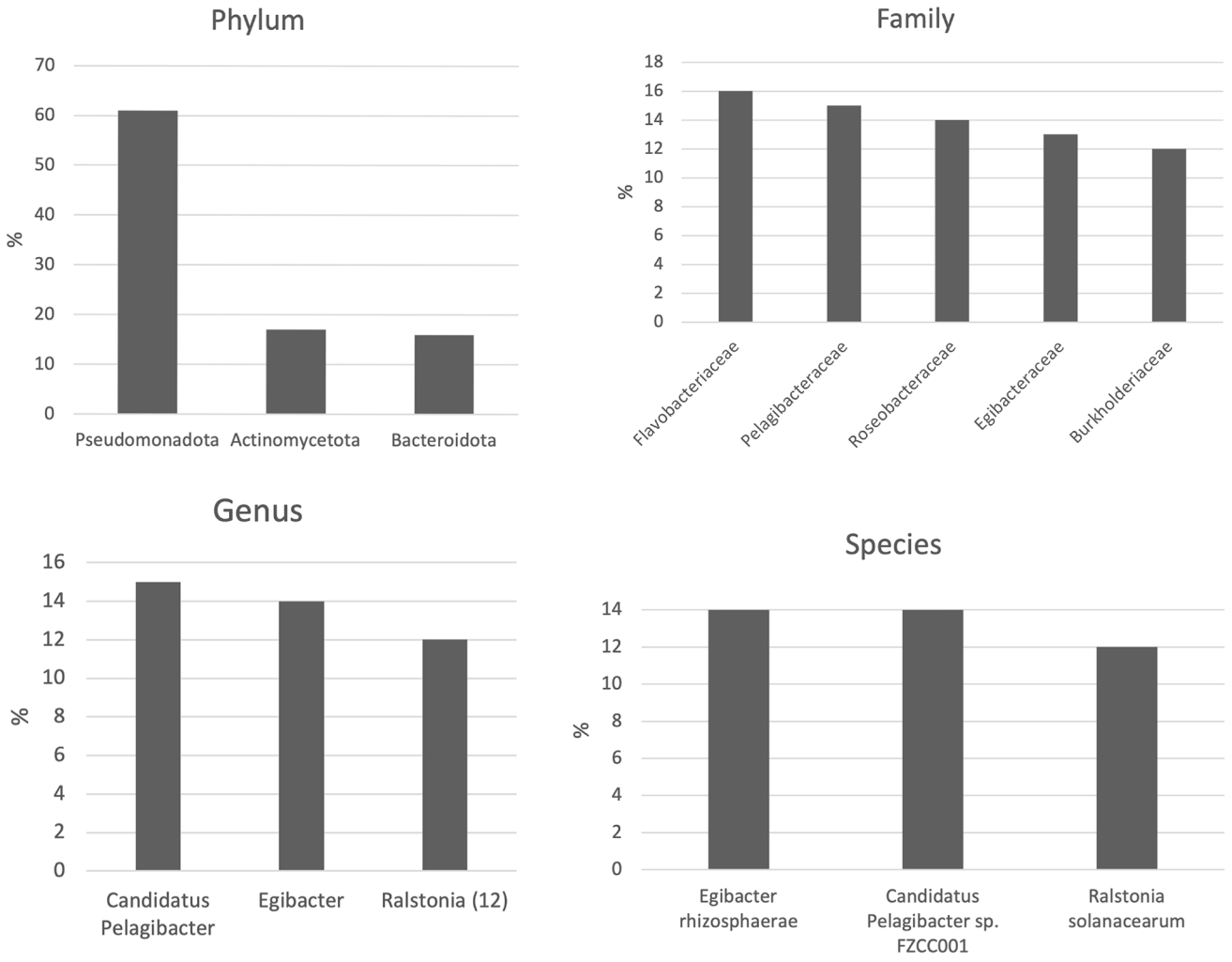

Figure 2. Shared taxa at the phylum, family, genus, and species level between the three samples. Only the taxa with relative abundances above 10% are sgown.

Pelagibacter sp., and 37 related to *Ralstonia* gen. and *Ralstonia solanacearum*. No article was found for *Egibacter* or *Egibacter rhizosphaerae*. Additionally, no review article was published about the ecosystem services of these taxa in the defined period of search in the systematic analysis. The relevant articles found follow as: 14 for *Pelagibacter* gen. and *Pelagibacter* sp., and 11 for *Ralstonia* gen. and *R. solanacearum* (Table 3).

Conclusion

Rivers present a key importance for human life and for nature balance since it is the primordial source of renewable water (Vörösmarty *et al.*, 2010). However, there is a lack of studies that focus the microbial diversity in rivers compared to marine or lake ecosystems that could elucidate about its relevance for urban areas and ecosystems services. The dynamic flow of water and nutrients in rivers make their study complex but necessary to answer foundational questions regarding land use and novel threats like antimicrobial resistance and the emergence of invasive species (Reddington *et al.*, 2020; van Rossum *et al.*, 2015). Despite the low number of samples analysed through this Brief Report, it is clear that we have insufficient information about the taxonomy of the most abundant taxa detected like *Pelagibacter* and *Egibacter* and more research is necessary to shed light on their relevant phenotypes aiming a better comprehension of the Tagus River ecosystem. Interestingly, the most abundant *Pelagibacter* strains were commonly found in marine waters rather than freshwaters ("FZCC0015" and "IMCC9063"). This observation constitutes a finding that deserves better attention since it may represent evidence of salinity changes in the Tagus River estuary.

Table 3. Relevant ecosystem services present in literature for the most abundant taxa.

TaxaID	Relevant reference	Ecosystem service/ phenotype
Candidatus *Pelagibacter* or SAR11	Rivers *et al.*, 2013	Re-establishing the pre-oil spill microbial community structure.
	Tsementzi *et al.*, 2019	Oligotrophy; auxotrophy for reduced sulfur (lacking a sulfite oxidase); differential phage predation and/or adaptation (fine tuning) of core genes to environmental conditions (e.g. temperature).
	Liu *et al.*, 2023	Resistance to salinity; ability to grow in low nutrients sites (like dissolved inorganic nitrogen); utilizers of dissolved organic carbon.
	Han *et al.*, 2020	Heterotrophy; carbon cycling; generalista; dimethylsulfoniopropionate degradation.
	Cerro-Gálvez *et al.*, 2021	Oligotrophy; antitoxicity strategies (to anthropogenic dissolved organic carbon; high tolerance to hydrophobic chemicals
	Chun *et al.*, 2021	'Skeleton'-formation and participate in species-engineered microenvironments; dominant in surface waters under stratified summer conditions; fine-tuning microbial modular structures; present in the nanoparticle-associated fraction; nitrate reduction, nitrification, dark sulfide oxidation.
	Delpech *et al.*, 2021	Oligotrophy; follow; abundant in low nutrient concentrations and in stratified surface waters phytoplankton blooms
	Sun *et al.*, 2021	Able to maximize nutrients uptake even when in low concentrations.
	Zhang *et al.*, 2022	Present in anticyclonic eddies, in warmer surface water temperatures, where there is less diverse bacterial communities; oligotrophy; survives at lower salinity and chlorophyl a concentrations but tolerate higher concentrations.
	Anderson & Harvey, 2022	Oligotrophy; rely on copiotrophs to assimilate sources of carbon and other metabolites.
	Fortin *et al.*, 2022	Predominant in *Alexandrium* sp. blooms; present in the particle-attached fraction of the surface water.
	Guo *et al.*, 2022	Involved in nitrogen and/ or sulfur cycling: prevalent in oxygen-rich surface oceans
	Larkin *et al.*, 2023	Heterotrophy; partition marine niche spaces; adapted to regions with low inorganic nitrogen supply; reveals habitat-specific partitioning in replication strategies for surface ocean communities
	Sun *et al.*, 2023	Prefers nitrogen-containing carbohydrates, oligo-glucans, oligo-cellulose, and lignin-derived aromatic fragments; polysaccharide degradation; scavenging mode in free-living fractions for the assimilation of dissolved carbohydrates, oligotrophy; key in significant flux of dissolved organic carbon and nutrient mineralization at the bottom of watery ecosystems.
Ralstonia	Coll *et al.*, 2020	Degradation of contaminants (e.g., organic pollutants);
	Rasool *et al.*, 2020	Tolerance to heavy metals like thallium; role in distributing metals; dominant in midstream soil samples; tent to enrich in bank of steam soils
	Chang *et al.*, 2021	Rare pathogenicity and transmitted through polluted waters.
	Liu *et al.*, 2023	Degrade microcystins; may enhance host-resistance to toxic cyanobacterial stress by affecting the whole bacterial community.
	Yang *et al.*, 2023a	May utilize diesel-derived organic intermediates produced by hydrocarbon degraders.
	Yang *et al.*, 2023b	Thrive in soil discharge and plant decomposition conditions; decomposition of several aromatic hydrocarbons.
R. solanacearum	Planas-Marquès *et al.*, 2020	Vascular pathogen; wide geographic distribution; colonizes many hosts, persists in soil and water for long periods; moves horizontally through the xylem ring by degrading the cell wall of primary xylem or pit membranes in the secondary xylem vessels.
	Biosca *et al.*, 2021	Infects ornamental plants; lytic lifestyle.
	Wen *et al.*, 2023	Cause wilt disease; affects the composition and diversity of rhizosphere microbiome

Ethics and consent

Ethical approval and consent were not required.

Data availability

BioProject Data Bank: 16S rRNA genomic data from the analyses of freshwater samples collected in Praínha do Braço de Prata (Lisbon, Portugal). Accession number: PRJNA1116853; https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA1116853; available in the NCBI Sequence Read Archive (Pedroso-Roussado *et al.*, 2024).

Contributorship CRediT statement

Cristiano Pedroso-Roussado: Conceptualization, Methodology, Investigation, Visualization, Writing – Original Draft, Writing – Review & Editing. **Mariana Pestana:** Validation, Writing – Review & Editing, Funding acquisition. **Ricardo Dias**: Methodology, Investigation, Data curation, Visualization. **Mónica Nunes**: Methodology, Investigation, Data curation, Visualization. **Pedro Pascoal**: Methodology, Investigation, Data curation, Visualization. **Marcelo Pereira**: Methodology, Investigation, Data curation, Visualization. **Nuno Nunes:** Validation, Writing – Review & Editing, Supervision.

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Open Peer Review

Current Peer Review Status: ? ? ✓

Version 3

Reviewer Report 22 April 2025

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✓ **Sushanta Deb**
Washington State University, Pullman, WA, USA

The manuscript has been substantially improved from its previous version, with each of the concerns raised thoroughly addressed. It can now be accepted.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Sequence analysis , metagenomics and comparative microbial genomics

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 2

Reviewer Report 24 January 2025

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? **Anyi Hu**
[1] Institute of Urban Environment, Chinese Academy of Sciences, Xiamen, China
[2] Institute of Urban Environment, Chinese Academy of Sciences, Xiamen, China

Thank author's efforts for addressing my comments. One of my major concerns related to the occurrence of Pelagibacter was well explained in the revised version. However, I think it would be

better to provide some environmental parameters, such as salinity etc., which can show the investigated area was under the influence of sea intrusion. By the way, the information of the introduction and conclusion sections, which highlighted the ecosystems service of ocean and river, are different. Based on the findings, I suggest may focus on the role of estuary and estuarine microbiome for ecosystem service.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Aquatic microbial ecology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 23 January 2025

https://doi.org/10.21956/openreseurope.20599.r49898

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Sushanta Deb
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Manuscript titled "Tagus River microbial profile through nanopore sequencing on samples gathered from Prainha do Braco de Prata, Lisbon" provide valuable information about microbial composition of freshwater ecosystem. However manuscript need attention to following points before indexing.

please mention how low quality reads were excluded from the raw sequence dataset.

Provide a detailed explanation of the bioinformatics method, including how clean reads were mapped to the database and which database was used for taxonomic assignment.

Replacing Table 3 with a bar chart depicting the microbial composition of the three samples would provide a more comprehensive and visually engaging presentation for the reader.

Please discuss the taxons identified at higher levels in these three samples in the context of published studies on the microbial community composition of freshwater rivers.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and does the work have academic merit?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?
No

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Sequence analysis , metagenomics and comparative microbial genomics

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Version 1

Reviewer Report 19 August 2024

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Anyi Hu
[1] Institute of Urban Environment, Chinese Academy of Sciences, Xiamen, China
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This report employed the third-generation (nanopore) sequencing to profile the taxonomic composition of bacterial communities in three surface water samples of the Tagus River. Candidatus *Pelagibacter*, *Egibacter*, and *Ralstonia* were identified as the main bacterial taxa, which were proposed to play an important role in maintaining the river ecosystem service (e.g., organic matter decomposition).

This work is a preliminary descriptive study due to the limited sample size and shallow analysis. However, this report provided a snapshot for the taxonomic information of the planktonic

bacterial communities in the Tagus River. Maybe in the future, more samples, more environmental variables and more advanced technologies (e.g., metagenomics) can be included into the Tagus River microbiome study.

I have a concern on the discussion about the ecosystem service of *Pelagibacter.* Since *Pelagibacter* is a typical marine bacteria, please carefully confirm whether the riverine bacteria is a marine one or the freshwater taxa who has a closed relationship with *Pelagibacter.* In addition, I think the current dataset and analysis of this report is hard to infer the ecosystem service provided by planktonic bacteria.

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and does the work have academic merit?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Not applicable

Are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Aquatic microbial ecology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 13 August 2024

https://doi.org/10.21956/openreseurope.19531.r42629

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Dave R. Clark
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The study by Pedroso-Roussado and colleagues characterises the microbial community structure of three water samples from the estuarine part of the Tagus River, Portugal. Although this is a low number of samples for a typical microbial ecology study, the authors have not over-interpreted their data nor conducted any under-powered statistical analysis. The authors report on the most abundant taxa detected, and infer their roles in organic matter degradation, highlighting the need for further data to better understand the role of understudied taxa. The study appears interesting, but some further basic information is required, particularly in the methods and results to provide further context.

Abstract:
Generally fine, but a few examples of odd / unclear phrases that may need revision. For instance, it is not clear what the authors mean by “Freshwater ecosystems play a vital role for humans and more-than humans”. If the authors mean that freshwater ecosystems are globally important, then it is better to say this in simple terms. It is also not clear what the authors mean by ‘consensual microbial profile’ – some re-wording is needed here.

Introduction:
Again, generally good, although it might have been nice to have a little more background on the Tagus River and why it is worthy of further study, as the introduction is a little too focussed on purely marine science rather than estuarine environments.

Methods:
There are some missing details on the molecular methods here. For example, how was DNA extracted from the samples? Were 16S genes amplified by PCR, if so, which primers?

Some additional details in the bioinformatics are also needed: “The used approach had been validated through ZymoBIOMICSTM Microbial Community Standard” – how was this approach validated and what data do the authors have to show this approach works? Also, to what sequencing depth were the samples rarefied to? Also, what steps are involved in this approach? At the moment, this methodology is not reproducible.

Results:
Again, there is some basic but interesting information about the samples missing. What was their diversity? How similar (beta diversity) were the samples to each other?
Also, whilst the literature search on ecosystem function was useful, why did the authors not use a bioinformatic tool to do this (e.g. PiCRUST2 or similar)?

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and does the work have academic merit?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Partly

If applicable, is the statistical analysis and its interpretation appropriate?
Not applicable

Are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Microbial community ecology, bioinformatics, statistical ecology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 25 Nov 2024

Cristiano Pedroso-Roussado

The authors want to thank Dr Dave Clark and Dr Anyi Hu for their kind revisions of your Brief Report.

We took into consideration all their comments and proceed to address them in full throughout this new version.
In summary, we remade the methods' section and now we believe it fully shows how the experimental work was conducted.
We greatly appreciated the comment of Dr Hu about the Pelagibacter marine or freshwater provenance. We checked in the dataset and indeed the two strains are described as marine bacteria, while here they were detected in estuarine Tagus River samples. This observation raises questions about the ecosystem dynamics of the Tagus River, and it deserves further exploration.
Finally, we hope that our response is of reviewers' and editors' appreciation and that our Brief Report can now be approved.
Thank you again

Competing Interests: none