Convocatoria AEET-SIBECOL de ayudas a proyectos de investigación ERC en ecología (12ª ed., 2022)

1. Datos de identificación.

Título de la propuesta	ALLWin: studying ***A**cacia* ***l**ongifo**l**ia* Green **W**aste Compost **in**corporation on soil microbial communities
Categoría	Tomando la iniciativa
Nombre y apellidos del Beneficiario	Joana Guedes de Jesus
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Departamento/Instituto/Grupo de Investigación/Otros	FCUL (Faculdade de Ciências da Universidade de Lisboa) and cE3c (Centre for Ecology, Evolution and Environmental Changes) & CHANGE (Global Change and Sustainability Institute)
Dirección, código postal, provincia	Rua Dr. Mário Amaral, nº48, r/ch esq. Murtal; 2775-124 Parede, Lisboa, Portugal

2. Memoria Técnica. Actividades y resultados de investigación

2.1. Introducción (Planteamiento, objetivos y justificación)

Biological invasions provide an opportunity to study plants' plasticity and adaptative capacity. *Acacia longifolia* Andrews (Willd.) is one of the most aggressive invaders worldwide, mainly distributed in Mediterranean-type climates. This species deeply interferes with soil environment due to its ability to establish symbioses with bacteria, altering soil pH and nutrient and water cycles (Marchante *et al.*, 2008; Ulm *et al.*, 2019). The extent of its impact on co-existing native species, especially regarding soil-plant-microbiota interactions and ongoing dynamics, remains unclear. Current control measures, such as, seedling hand pulling, cutting, chemical control (Wilson *et al.*, 2011; Duarte *et al.*, 2023), are largely inefficient, mostly due to long-term legacy effects and a persistent soil seed bank (Marchante *et al.*, 2009; Souza-Alonzo *et al.*, 2017); in this sense, alternative strategies are imperative for the local communities and ecosystems' conservation. Given the widespread invasion by *A. longifolia*, there is ongoing research into using it as Green-waste compost (Gwc) as a valuable source of organic matter (Brito *et al.*, 2013, 2015; Ulm *et al.*, 2022). In this sense, its application to regenerate degraded soils particularly those disrupt by forest fires, which is a raising concern in the Mediterranean considering more frequent and unpredictable forest fires. Altogether, the possibility of composting *A. longifolia* abundant biomass could both decrease this species occupied area and at the same time regenerate soils (Fig. 1).

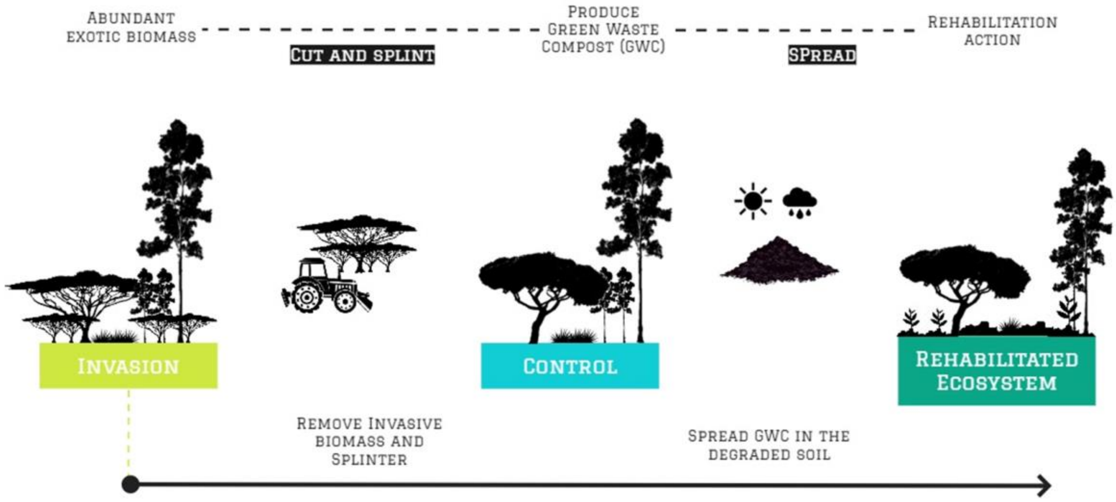

Figure 1 *Acacia longifolia* Green-waste compost (Gwc) process, since production until ecosystems' rehabilitation application.

This study aimed to understand how the incorporation of *A. longifolia* Gwc into soils influence its chemical properties and to characterize the microbial communities. The investigation focuses on the rhizospheric communities of three crucial native plants (*Arbutus unedo*, *Pinus pinea*, and *Quercus suber*), by assessing their aboveground development and fitness. Ultimately, the goal was to identify plant species that are more compatible with *A. longifolia* Gwc, contributing to more efficient forest management.

2.2. Descripción de la ejecución- Metodología

The methodology was applied as planned:

A field site already settled was used to do the Autumn sampling in October 2022, after the first precipitation event (as detailed in the proposal). This experimental setup is located in Lagos, Algarve, Portugal, in a private area burnt in June 2020, located within an ecosystem with Mediterranean climate (Csa, Köppen climate classification), characterized by sclerophyllous vegetation. One year after the fire, 10 x 10 m plots were implemented with machine-made swales 40 cm in depth (control plots, hereafter C), and some swales were filled with *Acacia longifolia* Green-waste compost (hereafter Gwc). This compost was produced using plant material (including trunks, branches, and leaves) sourced from ca. 1 ha of a monospecific *A. longifolia* area. The biomass was piled up under natural climatic conditions, and kept moist for one year to facilitate the composting process.

One year after field preparation (February 2021), three native plant species were planted in a reforestation action, including *Arbutus unedo*, *Pinus pinea* and *Quercus suber*. For this study, the two treatments were assessed: Gwc and C.

Rhizospheric soils from five individuals from the three plants species (15 samples *per* treatment, 5 *per* species) were sampled; in each individual, composite samples in a four-square method were collected using a core (5 cm x 10 cm in depth) for C and Gwc. Soils were deep-frozen (stored at -80 ºC) until further processing. Alongside, plant fitness parameters were assessed such as height, leaf reflectance (using Unispec-SC Spectral Analysis System, PPSystems) and chlorophyll fluorescence (using Handy PEA, Hansatech Instruments) (the latter two including five measurements *per* individual).

For Next-Generation Sequencing (NGS, Oxford Nanopore technology), soil samples collected underneath *A. unedo*, *P. pinea* and *Q. suber* were combined as a composite sample (5 individuals combined to represent each plant species) to cover the maximum representative diversity, for the two treatments (C *versus* Gwc) and for the two seasons (Spring and Autumn). Samples collected during the spring campaign (April 2022) have been collected but not analysed yet, whereas samples collected in the Autumn campaign (November 2022) were analysed using the funds from this call.

We end up with 12 composite samples that were analysed, targeting the soil microbiome: 16S and 25-28S rRNA regions for bacteria and fungi sequencing, respectively. This sequencing analysis was performed in BioISI Genomics ©. Briefly, DNA extraction was performed according to Yuan *et al.* (2019) and quality and purity were inferred through Qubit dsDNA high-sensitivity assay (v 1.01, ThermoFisher Scientific) and Nanodrop™ 2000 (ThermoFisher), respectively. After sequencing, data were carefully processed and analysed through a series of critical steps to ensure the accuracy and reliability of the results. After the removal of low-quality reads (lower than 1200 bps and higher than 1700 bps), the remaining reads were further filtered using Prinseq-lite version 0.20.4 (Schmieder and Edwards, 2011). This rigorous filtering ensured that only high-quality and informative reads were used in downstream analyses. The taxonomic classification of the preprocessed reads was performed using Kraken 2 (version 2.1.2), a powerful bioinformatic tool known for its fast and accurate taxonomic classification of DNA sequences, in accordance with Wood et al. (2019). In this classification, a comprehensive reference database was used, which included the NCBI RefSeq reference genomes and NCBI GenBank reference sequences of Bacteria and Fungi.

2.2.1 Data analysis

Non-Metric MultiDimensional Scaling (NMDS) based on the bacterial and fungal Operational Taxonomical Units (OTUs) identified for each plant species was performed to check the segregation according to the studied variables: Season (Spring and Autumn) and Treatment (C and Gwc).

Plant growth and rhizospheric soil physico-chemical parameters were tested by comparing means considering each Treatment within each Season, as well as each Season within each Treatment through Wilcoxon-Mann-Whitney test.

Data analysis was performed using the packages rstatix (Kassambara, 2023) and vegan (Oksanen et al., 2022) in R studio v 2023.09.0 (R Core Team, 2023).

2.3. Resultados obtenidos (cumplimiento de objetivos)

The results showed a significantly higher plant growth for all the studied plant species when Gwc was incorporated in soils (Fig. 2), highlighting for a higher development found in Autumn samples, inferred by a significant greater growth increment. This corroborates the initial hypothesis of using this Gwc to make positive soil amendments, ensuring high plant nutrition, ultimately improving plant fitness and survival.

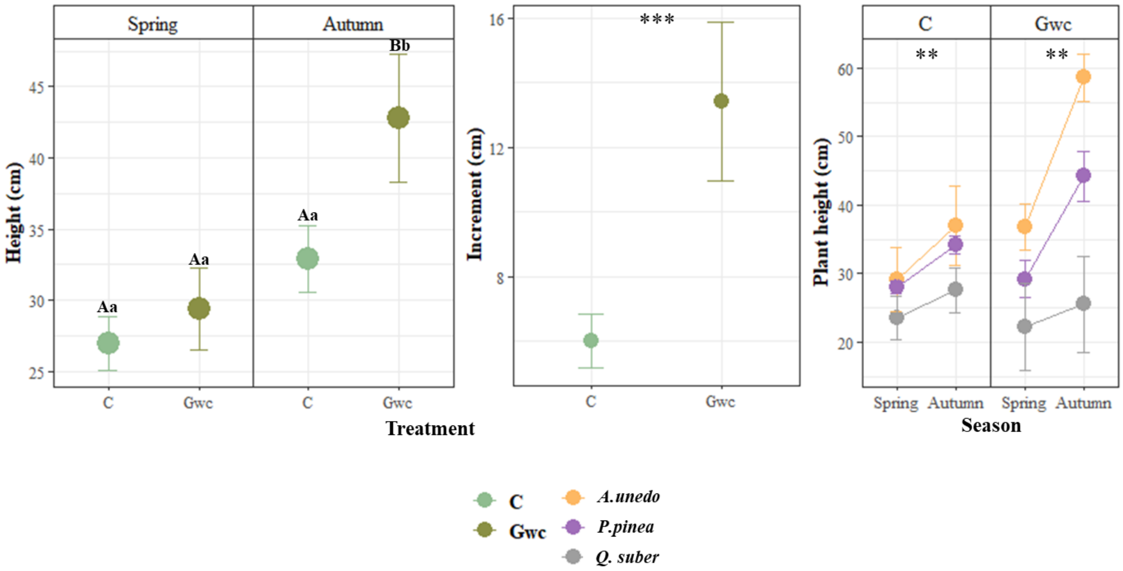

Figure 2 Height, increment (mean ± SE, n = 15, in cm) and plant growth (mean ± SE, n = 5; in cm) assessed for each plant species. Measurements were performed for both season, Spring and Autumn and under both studied treatments, C for Control and Gwc for *Acacia longifolia* Green-Waste Compost. Capital letters indicate significant differences between treatments within each season; small letters indicate differences between seasons within each treatment. * represents statistically significant differences.

Analyses of soil chemical parameters showed that there were differences between treatments within each season (C *versus* Gwc for Spring and Autumn, separately), and within treatments between seasons (C or Gwc in Spring *versus* Autumn) (Fig. 3). Indeed, this could explain the greater success of young saplings from *A. unedo*, *P. pinea* and even *Q. suber* despite this latter being a slow growing species.

In this sense, an increased vegetative growth measured in Autumn, determines that Gwc could have a long-lasting effect, allowing plant species to adapt more properly, making reforestation more successful, especially when associated with Gwc incorporation (Fig. 2, middle graph).

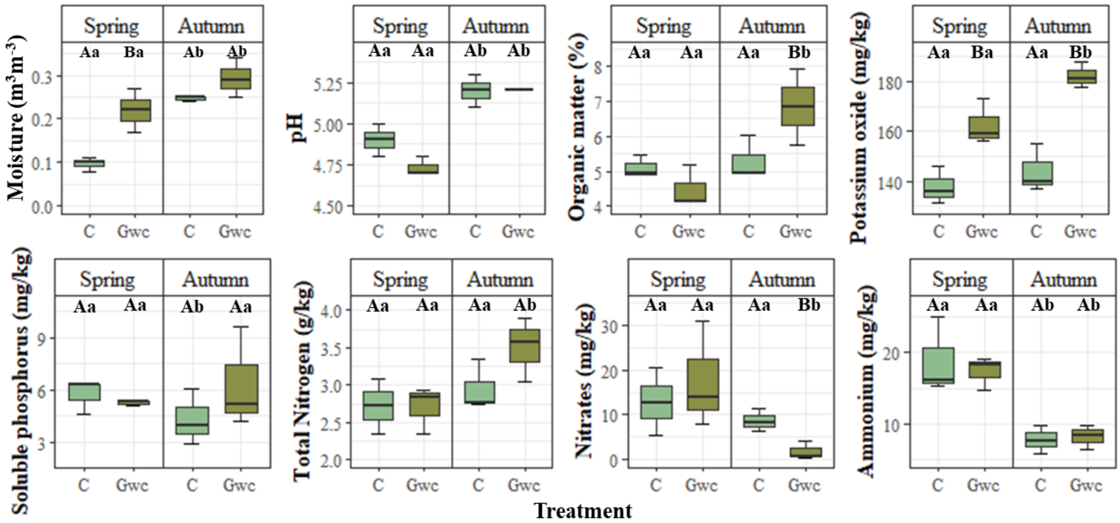

Figure 3 Rhizospheric soil parameters (mean ± SE, n = 15), namely, moisture (m^3m^{-3}), pH, organic matter (%), potassium oxide (mg/kg), soluble phosphorus (mg/kg), total nitrogen (g/kg), nitrates (mg/kg) and ammonium (mg/kg) assessed for two seasons (Spring and Autumn) and for the two studied treatments, C or Control (in blue) and Gwc or addiction of *Acacia longifolia* Green-waste compost (in green). Note that y axis is adjusted to each soil parameter. Capital letters indicate significant differences between treatments within each season; small letters indicate differences between seasons within each treatment.

Considering microbiome analysis, a segregation was found between the two studied treatments (C and Gwc) as well as for the two studied seasons (Spring and Autumn), for both bacteriome and mycobiome (Fig. 4).

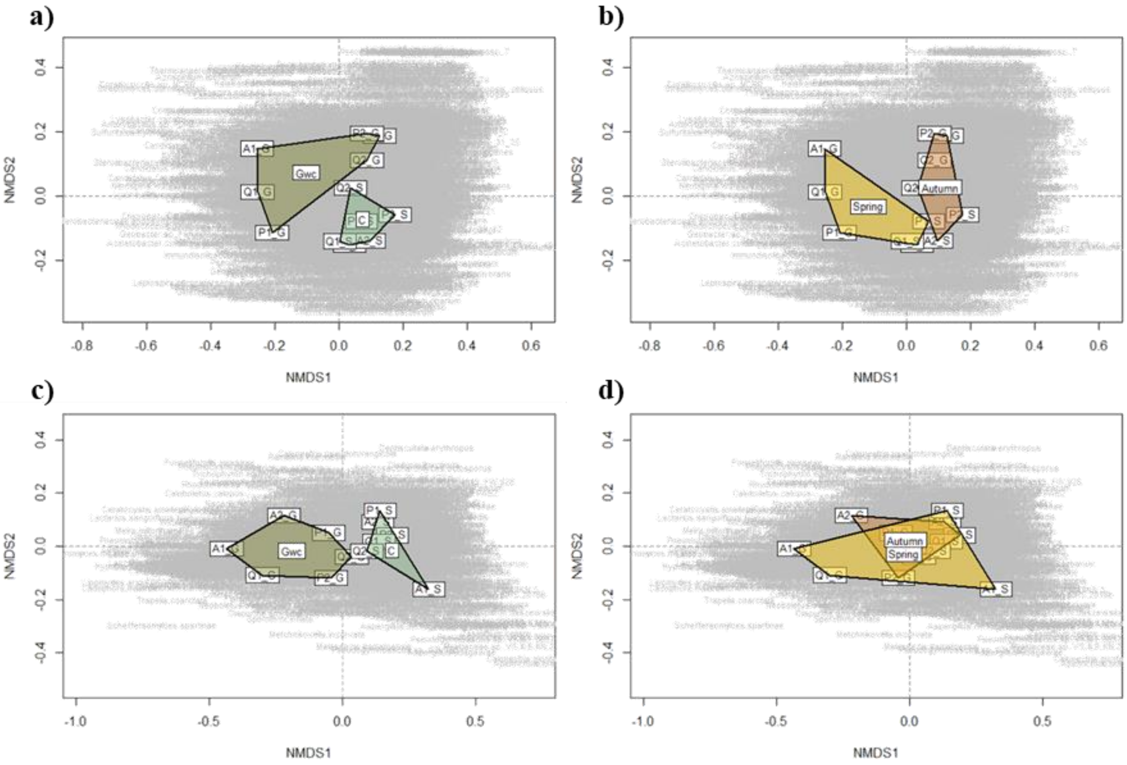

Figure 4 Two-dimensional Non-metric MultiDimensional Scaling (NMDS) ordination based on classified Operation Taxonomic Units (OTUs) for both microbial groups, bacteria (a and b) and fungi (c and d) (stress value of 8%) assessed for Season (Spring and Autumn) and Treatment (Control, C and Addition of *Acacia longifolia* Green-waste compost, Gwc). Colours represent different treatments and seasons: control (blue), Gwc (green), Spring (yellow) and Autumn (brown). Each plant species was considered for ordination and stands as follows: A, P and Q means *Arbutus unedo*, *Pinus pinea* and *Quercus suber*, respectively; 1 and 2 means Spring and Autumn, respectively; S and G means Control and Green-waste compost.

Regarding diversity, a core microbiome (both bacteria and fungi) shared to all plant species was identified. For example, *Stenotrophomonas* and *Pseudomonas* were the two bacterial genera more common and covering ca. 75 % of the top 10 relative abundance (Fig. 5). These genera are described as Plant Growth Promoting Bacteria (PGPB) being involved in different activities that benefit plant fitness (Santoyo *et al*, 2012; Kumar *et al.*, 2023). Also, since these two taxa are tolerant to extreme environments, allow them to survive better, being present regardless of treatment and season. In the case of mycobiome, *Cantharellus* was the only transversal genera. Being a mycorrhizal fungus, this genus mediates nutrient exchange with plant roots, aiding with water and mineral absorption at the same time (Bonfante and Genre, 2010) (Fig. 6).

Apart from this common core taxa, dominated by the genera mentioned above, other genera that change in abundance between plant species are also present, such as *Ktedonosporobacter*, *Paludibaculum*, *Bradyrhizobium* and *Conexibacter*, being described in literature as playing several roles in soil-plant interactions: i) taking part in different macro- and micronutrients uptake (nitrogen, phosphorus, sulphur and iron), ii) phytohormones' production stimulation and iii) antimicrobial activities (e.g., Majee *et al.*, 2018; de Albuquerque *et al.*, 2022). The same occurs with mycobiome, with *Saitozyma* and *Penicillium* changing in abundance, but being shared among plant species. These genera are, in spite of being from different phyla (Basidiomycota and Ascomycota, respectively), described in literature with antipathogenic activity against other fungal taxa, which could increase plant survival (Gorte *et al*., 2020; Ramos-Garza *et al*., 2023). In both cases, bacteriome and mycobiome, other genera are present but less transversally, suggesting some plant species specificity and selection.

Despite this, it seems that from Spring to Autumn, plants can shift partners and recruit different microorganisms according to their functional needs (given the increase in abundance), considering the top 10 taxa. Soil, especially the rhizosphere as the most active zone and the interface plant-soil, is an expression of microbiome functionality. In this sense, Gwc incorporation changes not only abundance, but also richness and diversity, indicators of potential multifunctionality, regardless of known microbial redundancy. This changes in the microbiome, highly related with changes in soil chemical properties, could promote plant adaptative capacity to better respond to climatic fluctuations, being more resilient – a crucial characteristic considering the ongoing (and fast) changing scenario, particularly in the Mediterranean forest ecosystems.

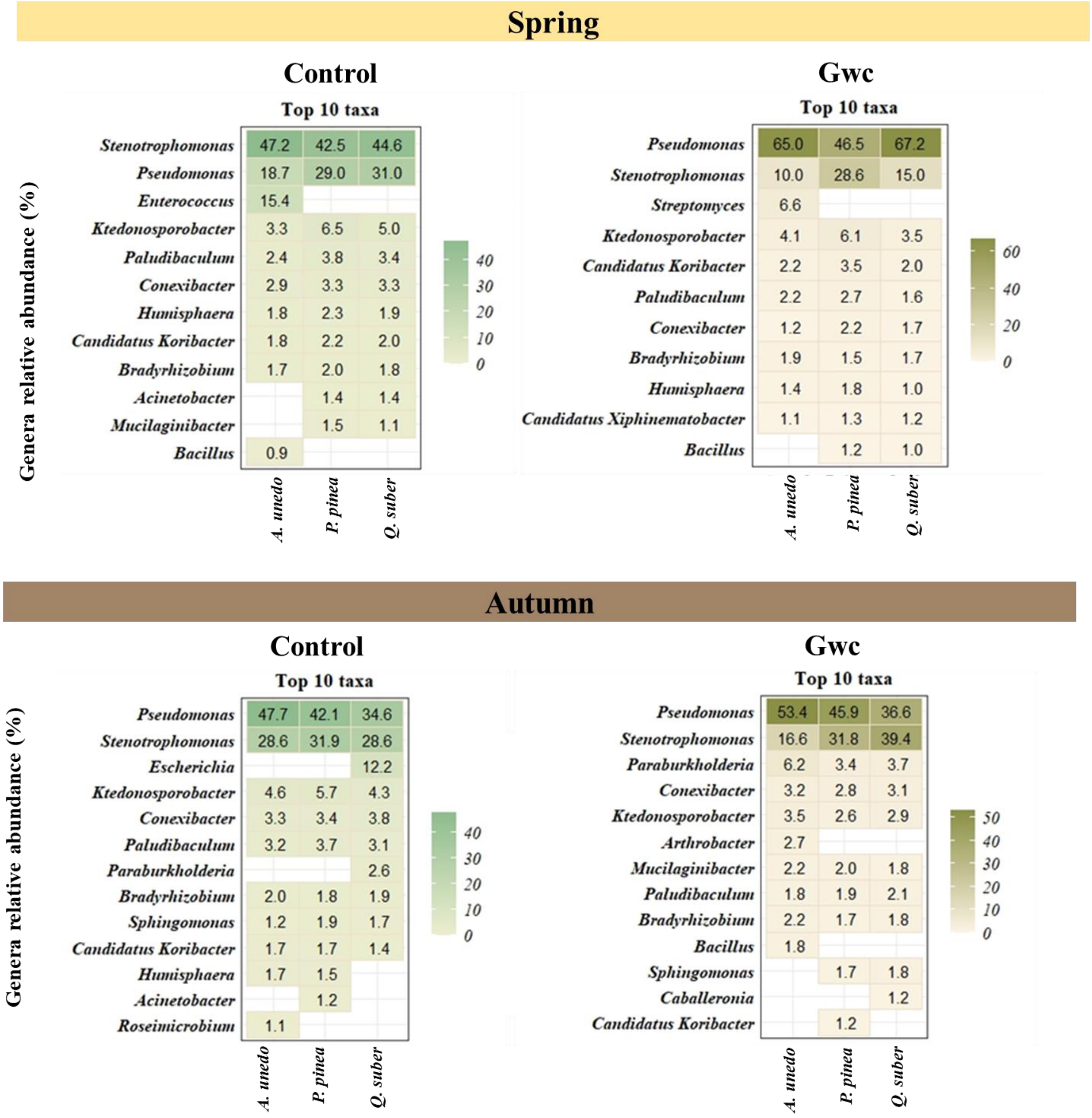

Figure 5 Top 10 heatmaps for bacterial genera (relative abundance, %) for both seasons, Spring and Autumn and for both treatments (C as control, in th left; Gwc, when *A. longifolia* Gwc was added, in the right) for the three studied species, *Arbutus unedo*, *Pinus pinea* and *Quercus suber*.

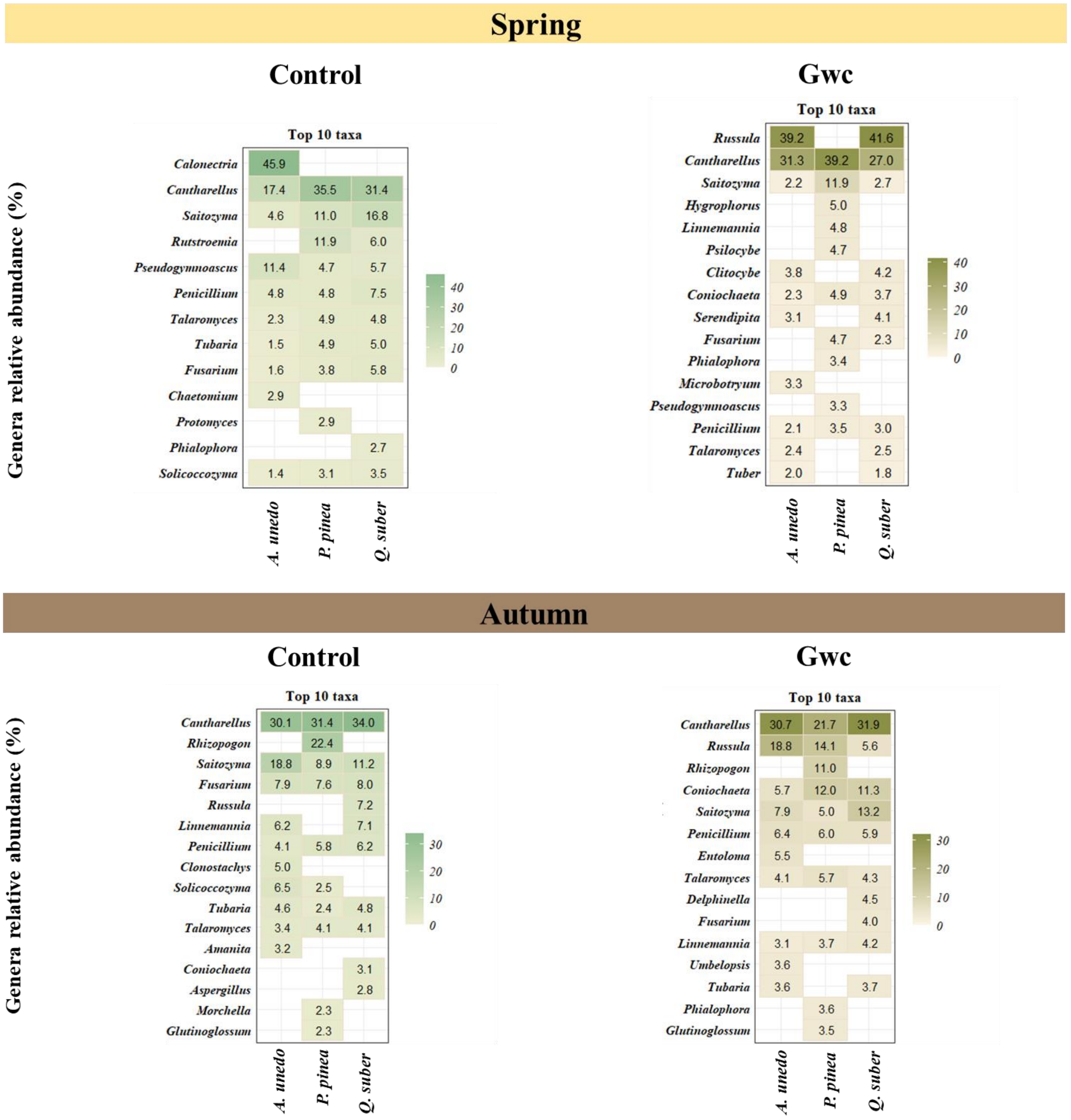

Figure 6 Top 10 heatmaps for fungal genera (relative abundance, %) for both seasons, Spring and Autumn and for both treatments (C as control, in th left; Gwc, when *A. longifolia* Gwc was added, in the right) for the three studied species, *Arbutus unedo*, *Pinus pinea* and *Quercus suber*.

2.4. Conclusiones y valoración de la ejecución

Altogether, data obtained allows to validate the possibility of using *Acacia longifolia* Gwc for forestry conservation. This Gwc improves soils and regenerates ecosystems' functionality and, the beneficial effects also create conditions for a more dynamic microbiome, making it more easily suitable for different plant species. Also, by using an experimental field site integrated in a Mediterranean region that collide with challenging predictions for climate and forest fires, the potentiality of this methodology is extensive and can be applied in a wide range of similar compromised forests. This study, undoubtably, gives a new perspective on invasive species associated with the raising concern on local biodiversity conservation. Giving another perspective, this data allows to point out that the effects that *A. longifolia* causes in soils as a living plant, can be taken in a composted form and used to recover soil fertility, altering also microbial communities' composition, doing an ecosystem-engineering that *A. longifolia* is being so known for.

Notwithstanding, long-term monitoring should be applied to understand how *A. longifolia* Gwc improved properties are still present and how the microbiome is adapting throughout time. Also, differences in richness and diversity for bacteriome and mycobiome could also be more evident when plant species are more developed, allowing a selection and a more strict/specific plant-microbe interactions.

The possibility of using Next-Generation Sequencing along with traditional soil methods allowed a more accurate conclusion, by assessing the maximum potential of microbiome analysis (*viz* culture-independent methods), that now can be explored for functionality rendering the known identified diversity (ongoing research). In this sense, this study contributes with new and practical knowledge to apply to have a more efficient soil rehabilitation combined with reforestation actions to regenerate native forests, in such a relevant region as the Mediterranean forests.

2.5. Publicaciones resultantes

The results obtained from this budget support were presented in the following scientific meetings:

- Conference TiBE 2023, Invaders in the Horizon – Advancing Invasion Science, from Genes to Ecosystems to Society, that occurred between 27th and 30th of November in Vairão, Vila do Conde, Portugal. The presentation was intitled "Using *Acacia longifolia* biomass to improve soils: another perspective on invasive plants", accepted as an Oral communication (Annex I);
- Project "INVASIVES: Biological invasions across terrestrial and marine habitats: histories, common traits, and ecological impacts" on the 19th of January in Faculty of Sciences of the University of Lisbon (FCUL), as a sustainable application of *Acacia longifolia* (as an invasive plant) biomass.

Also, a scientific publication is being prepared to be submitted in the first trimester of 2024 to submit in a scientific journal with impact factor (IF), having "Applied Soil Ecology" (IF 5.5) as first option followed by "Plant and Soil" (IF 4.9). The idea is to take advantage of the integration given by the results of parameters assess in the below- and aboveground, and all the techniques applied to draw the conclusions, rendering this subjacent new perspective on *A. longifolia* as an invasive plant.

Notes on the Budget:

In spite of this budget had been used for a some Next-Generation Sequencing analysis, this report includes the remaining data on Spring and soil chemical properties to better integrate the data obtained and to be more cohesive discussion.

Initially, the budget was planned to Sanger Sequencing (after following the classical microbiology procedures) along with Next-Generation Sequencing (culture-independent method). However, since the first part was already ongoing, funding was totally allocated for the second method (Annex II & III). It was a great advantage, taking into account soil as an incredibly diverse environment and the effort ascribed to classical cultivation methods.

Notwithstanding, classical microbiology was performed, however, as a case-study for Spring season (the first sampling date) and since it was already done, data is not included in this report. However, the plan stands for publishing also the results from this culture-dependent methods and integrate the NGS analysis for Spring, so acknowledgments will integrate AEET and this funding.

1. **Informe de gastos del proyecto.** Relación de partidas de gastos y sus importes. Se deberán aportar justificantes originales de los pagos realizados (tickets, recibos o facturas).

Please find the PDF attached as Annex IV.

Joana Guedes de Jesus

Fdo: Joana Guedes de Jesus

26 January 2024

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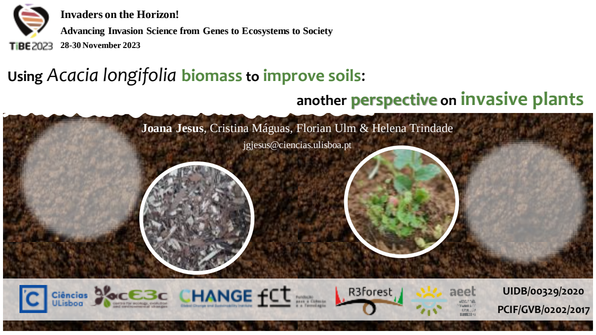
Annex I



Invaders on the Horizon!
Advancing Invasion Science from Genes to Ecosystems to Society
28-30 November 2023
TIBE 2023

Using *Acacia longifolia* biomass to improve soils:
another perspective on invasive plants

Joana Jesus, Cristina Máguas, Florian Ulm & Helena Trindade
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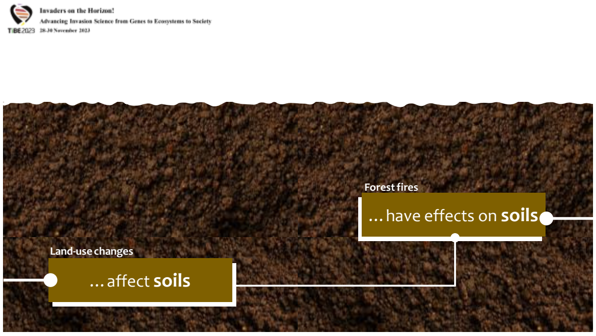

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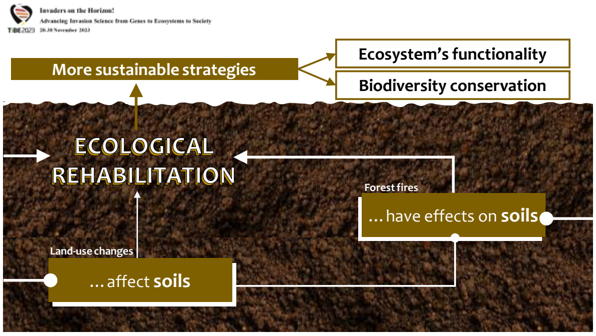

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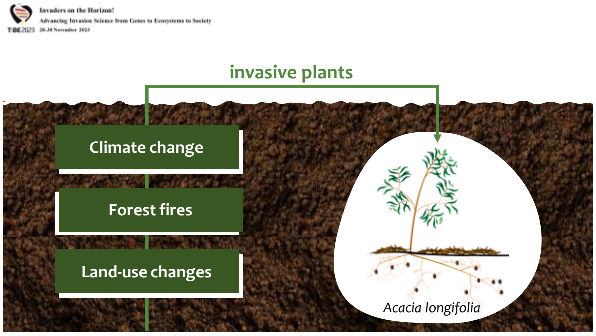

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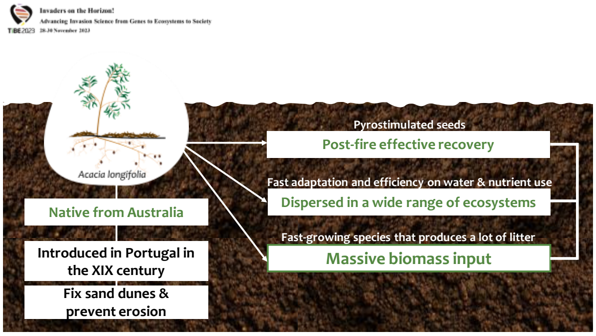

Invaders on the Horizon!
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Acacia longifolia

Native from Australia

Introduced in Portugal in the XIX century

Fix sand dunes & prevent erosion

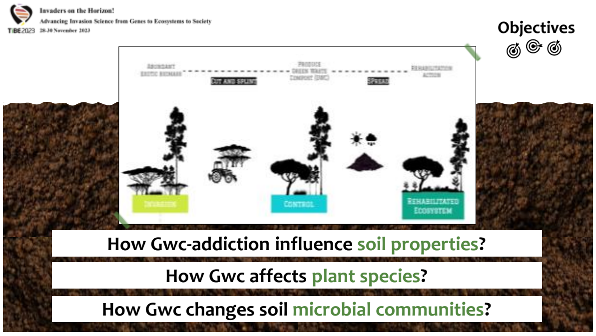
Pyrostimulated seeds
Post-fire effective recovery

Fast adaptation and efficiency on water & nutrient use
Dispersed in a wide range of ecosystems

Fast-growing species that produces a lot of litter
Massive biomass input

Invaders on the Horizon!
Advancing Invasion Science from Genes to Ecosystems to Society
TIBE2023 28-30 November 2023

What if *Acacia longifolia* biomass can be used to produce Green-Waste Compost (Gwc)?



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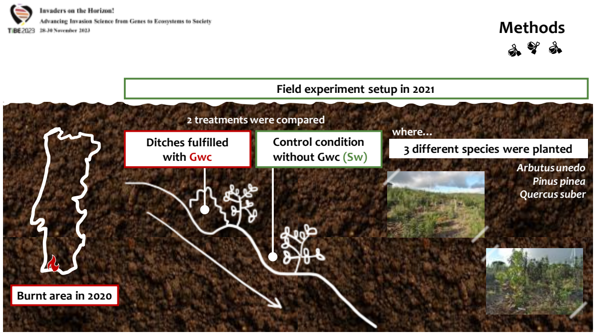
Objectives

How Gwc-addiction influence soil properties?

How Gwc affects plant species?

How Gwc changes soil microbial communities?



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Methods

Field experiment setup in 2021

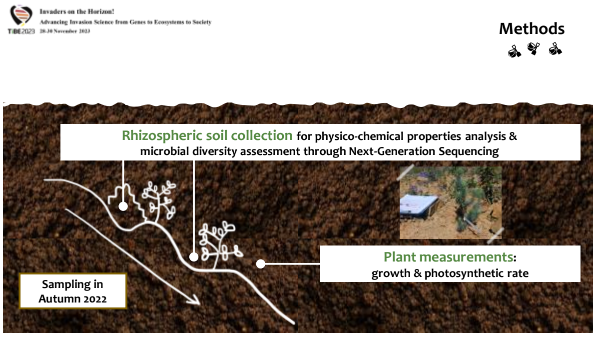
2 treatments were compared

Ditches fulfilled with Gwc

Control condition without Gwc (Sw)

where…
3 different species were planted
Arbutus unedo
Pinus pinea
Quercus suber

Burnt area in 2020



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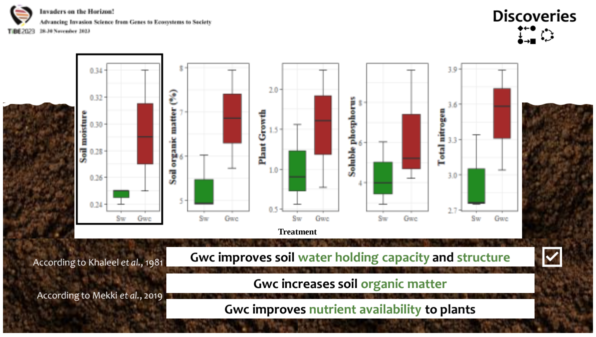
Methods

Rhizospheric soil collection for physico-chemical properties analysis & microbial diversity assessment through Next-Generation Sequencing

Sampling in Autumn 2022

Plant measurements:
growth & photosynthetic rate



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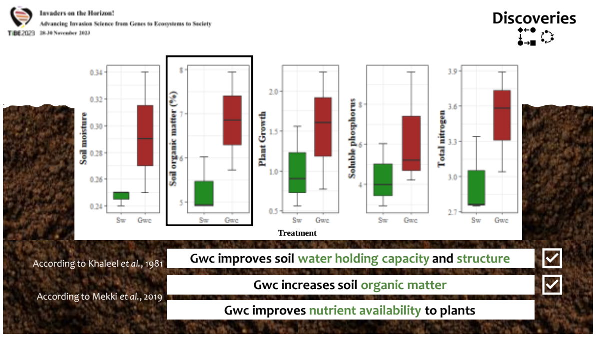
Discoveries

According to Khaleel *et al.*, 1981
Gwc improves soil water holding capacity and structure ☑

Gwc increases soil organic matter

According to Mekki *et al.*, 2019
Gwc improves nutrient availability to plants



Discoveries

According to Khaleel *et al.*, 1981 — Gwc improves soil water holding capacity and structure ☑

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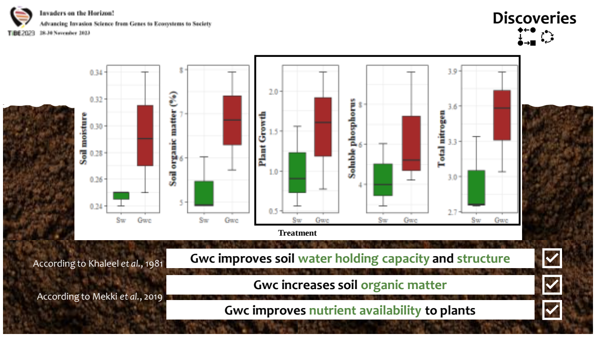

Discoveries

According to Khaleel *et al.*, 1981 — Gwc improves soil water holding capacity and structure ☑

According to Mekki *et al.*, 2019 — Gwc increases soil organic matter ☑

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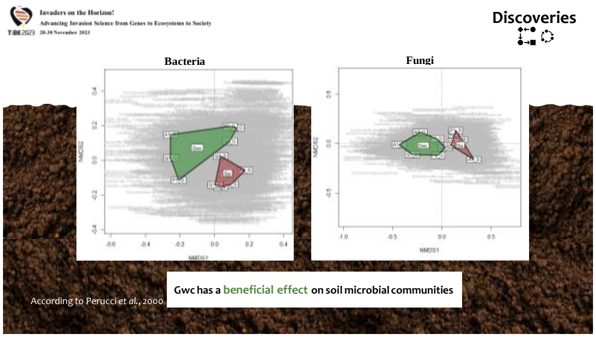
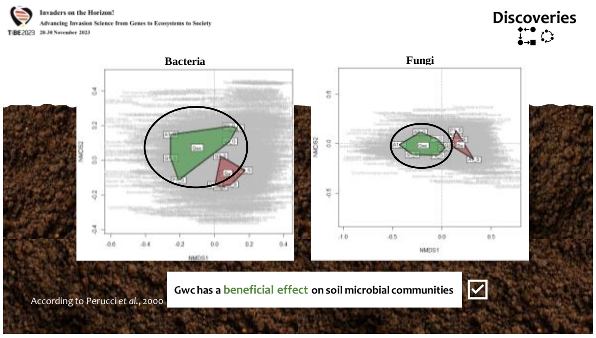


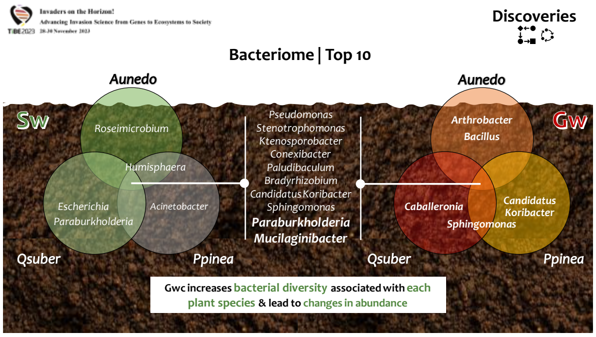
Discoveries

According to Perucci et al., 2000 — Gwc has a beneficial effect on soil microbial communities



Discoveries

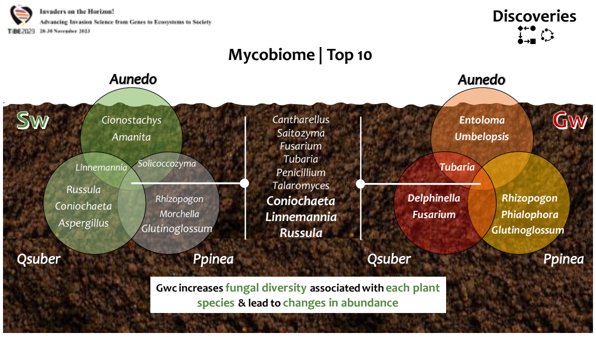
According to Perucci et al., 2000 — Gwc has a beneficial effect on soil microbial communities ☑



Discoveries

Bacteriome | Top 10

Gwc increases bacterial diversity associated with each plant species & lead to changes in abundance



Discoveries

Mycobiome | Top 10

Gwc increases fungal diversity associated with each plant species & lead to changes in abundance

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Conclusions

Acacia longifolia Gwc improves soil properties & quality and plant development

Gwc shifts soil microbial communities in diversity and abundance

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Thank you | Obrigada

Ciências ULisboa
fct Fundação para a Ciência e a Tecnologia
cE3c centre for ecology, evolution and environmental changes
CHANGE Global Change and Sustainability Institute

Annex II

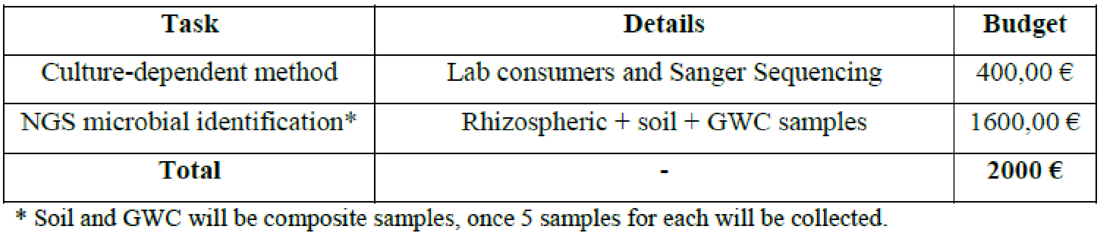
Lisboa 25 de julio 2023

Estimados Sres.

Por la presente solicito una alteración en la distribución de los gastos de la cantidad para mí asignada en el Convocatoria de Ayudas a Proyectos de Investigación de la Asociación Española de Ecología Terrestre en Ecología (AEET) (edición 2022) a través de Sociedade Portuguesa de Ecologia (SPECO), en la modalidad *Tomando la iniciativa*.

Solicito que la ayuda completa (2000 €) sea usada íntegramente en el análisis de secuenciación de próxima generación para garantizar resultados de calidad (reproducibles, replicables y sólidos) susceptibles de publicación.

Originalmente, la cantidad prevista se dividiría de acuerdo con la siguiente tabla:



Task	**Details**	**Budget**
Culture-dependent method	Lab consumers and Sanger Sequencing	400,00 €
NGS microbial identification*	Rhizospheric + soil + GWC samples	1600,00 €
Total	**-**	**2000 €**

* Soil and GWC will be composite samples, once 5 samples for each will be collected.

Sin embargo, ahora solicito que el valor total se destine íntegramente a la tarea de "NGS microbial identification", como descrito en la siguiente tabla:

Task	**Details**	**Budget**
NGS microbial identification	Rhizospheric + soil + GWC samples	2000€

Atentamente,

Joana Jesus

Joana Guedes de Jesus

aeet
ASOCIACIÓN
ESPAÑOLA DE
ECOLOGIA
TERRESTRE

Annex III

Attn: **JOANA GUEDES DE JESUS**

Asunto: Beca a proyecto: “*ALLWin: studying Acacia longifolia Green Waste Compost incorporation on soil microbial communities*”, con IP: Joana Guedes de Jesus.

La Dra. Cristina Armas, Secretaria de la Asociación Española de Ecología Terrestre (AEET) y responsable del área de Proyectos de Investigación.

Por la presente comunica a la interesada que,

atendiendo a su solicitud, presentada por escrito a esta Secretaría el día 27 de julio de 2023, de desviación de las partidas presupuestarias inicialmente declaradas en la memoria de su proyecto titulado: “*ALLWin: studying Acacia longifolia Green Waste Compost incorporation on soil microbial communities*”, que resultó beneficiario de una beca en la Modalidad “Tomando la iniciativa”, en la 12ª edición de la Convocatoria de concesión de ayudas a proyectos de investigación en ecología liderados por jóvenes investigadores”, año 2022 y, con motivo de la justificación alegada por la interesada, la Junta Directiva de la AEET, ha resuelto autorizar la desviación solicitada del presupuesto, que se dedicará íntegramente a análisis de secuenciación *NGS microbial identification*.

Y para que conste a los efectos oportunos y a petición del interesado, firmo la presente en Almería a 07 de julio de 2023.

Fdo.: Cristina Armas

AEET
ASOCIACIÓN ESPAÑOLA
DE ECOLOGÍA TERRESTRE
C.I.F. G-50359017

Annex IV

ATCUD: JFXPZ6TY-154

FCiências.ID - Ass. para a Inv. e Desenv. Ciências
Contribuinte N.º: 514187808

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www.fciencias-id.pt

Exmo.(s) Sr.(s)
Joana Guedes de Jesus
Rua Doutor Mário Amaral, nº49, R/C Esq.

Murtal
2775-124 Parede

Fatura FT FA.2023/154

Original

Fatura

V/N.º Contrib.	Requisição	Moeda	Câmbio	Data
257578714		EUR	1,00	2023-08-24

Desc. Cli.	Desc. Fin.	Vencimento	Condição Pagamento
0,00	0,00	2023-08-24	Pronto Pagamento

Artigo	Descrição	Qtd.	Un.	Pr. Unitário	Desc.	IVA	Valor
02	PCR testing for 16S and 28S rDNA gene	8,000000	UN	203,2520	0,00	23,00	1 626,02
	DNA extraction and quality control						
	16S and 28S profiling by 4th NGS Sequencing						
	Depth: 100k/Sample						
	Bioinformatic Analysis						

FaRy-Processado por Programa Certificado n.º 0030/AT / FT FA.2023/154 | Os bens e/ou serviços foram colocados à disposição na data 2023-08-24 / © PRIMAVERA BSS /

Quadro Resumo de Impostos

Taxa/Valor	Incid./Qtd.	Total
IVA (23,00)	1 626,02	373,98

Motivo Isenção

ATCUD: JFXPZ6TY-154

Mercadoria/Serviços	1 626,02
Descontos Comerciais	0,00
Desconto Financeiro	0,00
Portes	0,00
Outros Serviços	0,00
Adiantamentos	0,00
IEC/Outras Contribuições	0,00
IVA	373,98
Acerto	0,00
Total (EUR)	**2 000.00**

Dados Bancários

Banco: Banco BPI

IBAN: PT50001000005473601000139

SWIFT: BBPIPTPL