Convocatoria de ayuda a proyectos de investigación liderados por jóvenes investigadores (8º ed., 2018)

1. Datos de identificación

Título de la propuesta	Uncovering the **vol**atile **na**ture of **bry**ophytes **VolNaBry**
Nombre y apellidos del solicitante	RAQUEL ESTEBAN TERRADILLOS
Datos de contacto: e-mail y teléfono	raquel.esteban@ehu.eus; 946013229
Departamento/Instituto/Grupo de Investigación/Otros	Departamento Biología vegetal y Ecología Universidad del País Vasco (UPV/EHU)
Dirección, código postal, provincia	c/Sarriena s/n 48080 Bilbao Bizkaia

2. Memoria Técnica. Actividades y resultados de investigación

2.1. Introduction

Approach and the state of the art

Biogenic volatile organic compounds (BVOCs) are produced by plants for key ecophysiological processes, ranging from modifying the stress tolerance (Vickers *et al.*, 2009) to influencing trophic interactions as infochemicals (Peñuelas & Staudt, 2010). BVOCs are also involved in chemical processes within the atmosphere that influence climate change in several ways, as they are main biogenic precursors of ozone (Peñuelas & Staudt, 2010) and recently they were related with clouds formation over forest regions (Teuling *et al.*, 2017). Current inventories of BVOC emission cover more than 1200 plant species but do not show any clear phylogenetic pattern (Loreto & Fineschi, 2015). Remarkably, observations about synthesis and emission of BVOCs in bryophytes are scarce, not recent, and mainly focussed on isoprene (the main BVOC emitted by plants)(Hanson *et al.*, 1999). Isoprene emission is an ancestral trait that was often lost when plants colonized the terrestrial environment (Loreto *et al.*, 2014). It was then reported in phytoplankton adding support to this idea (Srikanta Dani *et al.*, 2014). Bryophytes were the first land colonizers, so they may be the ideal model to test such hypothesis. Although the bryophyte biomass is relatively low, considering the large surface area covered by these organisms and their importance in community structure and ecosystem function, BVOC emission (if any) may considerably affect the global budget of biogenic volatiles and may have relevant consequences for biosphere structure and functioning.

The main objective was to characterize BVOC constitutive emissions by bryophytes across different species, ecological habitats and terrestrial biomes. This project links bryophytes emission traits to their latitudinal niches, hypothesising that it could favour the BVOCs emission differential in extreme weathers (Tropical, Subarctic) to less extreme (Mediterranean, Atlantic). For fulfil this general aim, I evaluated via infochemistry the following specific aims

i) To *investigate if bryophytes are important BVOC sources*
ii) To *cover bryophytes BVOC composition among phylogenetic groups*
iii) *To relate the type of BVOCs emitted with the bryophyte location, habitat or ecological requirements of the species*
iv) *To discover which is the possible function this emission in bryophytes?*

Justification

In order to fulfil these specific aims, a BVOCs screening in bryophytes (Bryophyta and Marchantiophyta) along two different scenarios (extreme *versus* template climate conditions) was performed, together with physiological measurements (details in methodology), during the year 2018. The bryophytes were the first land colonizers and their ecological importance in community dynamics is indisputable, thus this study will set light on the BVOCs evolution and functions in bryophytes. This proposal will be especially valuable under global warming scenario that may change the emission patterns in bryophytes with important consequences for future biosphere-atmosphere interactions.

2.2. Methodology

Locations and species selection

Bryophytes were collected in 6 different locations in collaboration with local scientists. See details in Figure 1 and Table 1.

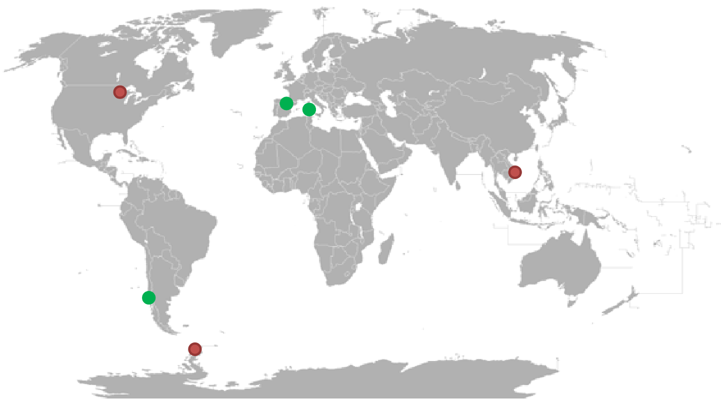

Fig. 1. Map of the locations used for sampling of bryophytes. Red and green dots indicate extreme and template climate conditions, respectively.

Table 1. Locations, where the bryophytes have been sampled. Additional information has been included regarding climate, type of biome, number of species collected, sampling dates and key collaborators in the task of sampling.

Location	Climate (based on Köppen-Geiger classification)*	Type of biome	Sampling dates	Number of species sampled	Collaborators
Minnesota, USA	Hemi boreal	Extreme	August September October	20	D. Stanton (Univ. Minnesota)
La Rioja, Spain	Template	Template	April	24	JM. Abaigar

	Atlantic and Mediterranean		May		and E. Núñez-Olivera
Cerdeña, Italy	Template Mediterranean	Template	April May	21	A. Cogoni, and P. Cortis
Katalapi Park, Llanquihue, Chile	Template Oceanic	Template	February	3	Jl. García-Plazaola
Bidoup , Vietnam	Tropical dry	Extreme	September	29	H. Truoung Luu, L. Buu Thach, N Thanh Luc (Vietnam Academy of Science and Technology)
Juan Carlos I base, Antártida	Polar	Extreme	February	6	A. Perera (Univ, Balearic Islands)

*(Kottek *et al.*, 2006)

In the initial VolNaBry proposal, Nicaragua and Finland were proposed as tropical and subarctic climate locations. Unfortunately, sampling in these locations was impossible, due to the political instability and conflicts in Nicaragua. In the case of Finland, the local finish collaborators withdrew the participation in the proposal due to lack of time. In order to replace the extreme climate locations, I contacted D. Stanton from the University of Minnesota and H. Truoung from Vietnam Academy of Science and Technology. This contingency plan incorporated the extreme locations (as was stated in the proposal) but delayed the field campaigns around 3 months, and consequently, all the chronogram was postponed to the end of the field campaigns.

The scientific criterion to select the species in each location was the following: (i) same species or genus or family, (ii) different representatives of liverworts and mosses, (iii) similar ecological requirements and (iii) the number of individuals over a given area to ensure that plant sampling does not have a significant impact on the population. In addition to scientific criteria, there were other aspects that were taken into consideration to build the final list. Among them, (i) species availability and accessibility (being abundant and non-endangered species) and (ii) other technical limitations such as unsuitable shape of photosynthetic structures (see Table 2 for details).

Table 2. Summary of the species collected across biomes

Genus	Orden	Liverwort (L) or moss (M)	Sampled in
Frullania	Jungermanniales	L	Spain, Vietnam
Lunularia	Marchantiales	L	Spain, Italy
Marchantia	Marchantiales	L	Spain, Vietnam
Pellia	Pelliales	L	Spain
Plagiochila	Jungermanniales	L	Spain

Porella	Hypnales	L	USA, Spain
Scapania	Jungermanniales	L	Italy, Spain
Antitrichia	Hypnales	M	Italy, Spain
Atrichum	Polytrichales	M	USA, Vietnam
Bartramia	Bartramiales	M	Italy, Spain
Bazzania	Marchantiales	L	Vietnam
Brachythecium	Hypnales	M	Italy, Spain, The Antarctic
Bryum	Bryales	M	Spain, The Antarctic, Vietnam
Calliegonella	Hypnales	M	Spain, USA
Campylotus	Dicranales	M	Chile, Vietnam
Ceratodon	Dicranales	M	Spain, USA, The Antarctic
Cratoneuron	Jungermanniales	M	Spain
Dendroligotrichum	Polytrichales	M	Chile
Dicranum	Dicranales	M	Spain, USA
Diphyscium	Diphysciales	M	Vietnam
Distichophyllum	Hookeriales	M	Vietnam
Fontinallis	Hypnales	M	Italy, Spain,
Hedwigia	Hedwigiales	M	Italy, USA
Herbertus	Jungermanniales	L	Vietnam
Homalothecium	Hypnales	M	Italy, Spain
Hypnodendrom			Vietnam
Hypnum	Hypnaceae	M	Italy, Spain
Leucodon	Hypnales	M	Spain
Leucobryum	Dicranales		Vietnam
Mnium	Bryales	M	Italy, Spain
Orthotrichum	Orthotrichales	M	Spain, USA
Norwellia	Jungermanniales	L	USA
Plagiomnium	Bryales	M	Spain, USA
Pleurozium	Hypnales	M	USA
Polytrichum	Polytrichales	M	Italy, USA
Polytrichastrum	Polytrichales	M	Spain
Pseudoscleropodium	Hypnales	M	Italy
Pogonatum	Polytrichales	M	Vietnam
Ptychostomum	Bryales	M	Italy
Pylasia		M	USA
Racomitrium	Grimmiales	M	Italy
Rhytidiadelphus	Hypnales	M	Italy, USA
Schistidium	Grimmiales		The Antarctic, USA
Sphagnum	Sphagnales	M	Italy, USA
Syntrichia	Pottiales	M	Spain
Thamnobryum	Neckerales	M	Italy
Thuidium	Hypnobryales	M	Chile

Trematodon	Dicranales	M	Vietnam
Trismegistia	Hypnales	M	Vietnam
Trichocolea	Jungermanniales	L	Vietnam
Warnsorfia	Hypnales	H	The Antarctic

Species field sampling

The collection was carried out according to a standardized sampling protocol. During the collection, bryological attributes were collected for the bryophytes based on Ellenberg et al (1991) and Hill et al. 2007 (eg. Table 3). Bioclimatic variables for each of the sampling locations have been obtained from WorldClim global database (www.worldclim.org). Microhabitat irradiance was characterized (only for some of the bryophytes, most of them the ones sampled in Spain). Solar radiation and canopy openness were estimated by analysis of hemispherical photographs with Gap Light Analyzer (GLA) software. This imaging software to extract canopy structure and gap light transmission indices from true-colour fisheye photographs (Fig. 2).

Table 3. Data for some of the bryophytes used in this study for the location of Spain. Nomenclature of species follows the Tropicos database (http://www.tropicos.org/). For each species, the following data are provided: sampling locality and country, including altitude (Alt), latitude (Lat) and longitude (Lon) (ETRS89 datum); type of forest; L, T, F, R, N and S, Ellenberg indicator values, respectively, for Light (from 1 -plants in deep shade- to 9 -plants in full sun), Temperature (from 1 -plants typical from extremely cold environments- to 9 -plants from extremely hot environments; x = indifferent), Moisture (from 1 -species from extremely dry environments- to 12 -normally submerged species), Reaction (pH; from 1 -species from extremely acid environments- to 9 -species from substrate with free calcium carbonate, mainly chalk and limestone), Nitrogen (from 1 -species from extremely infertile sites- to 7 -species often found in richly fertile places), and Salt tolerance (from 0 –species absent from saline sites- to 5 –species of saltmarshes or obligate halophytes). Ellenberg indicator values were obtained from Ellenberg et al. (1991) and Hill et al. (2007).

Species	Sampling locality	Alt (m)	Lat (ºN)	Lon (ºE)	Forest	L	T	F	R	N	S
Frullania tamarisci (L.) Dumort.	El Rasillo de Cameros (La Rioja)	1163	42.11	-2.42	*Pinus sylvestris*	7	3	4	5	2	1
Lunularia cruciata (L.) Lindb.	Nieva de Cameros (La Rioja)	947	42.13	-2.40	Riparian (Salicaceae)	4	8	7	7	7	0
Marchantia polymorpha L.	Nieva de Cameros (La Rioja)	947	42.13	-2.40	Riparian (Salicaceae)	7	6	9	6	4	0
Pellia epiphylla (L.) Corda	Lumbreras (La Rioja)	1030	42.08	-2.39	*Pinus sylvestris*	4	4	8	4	4	0
Plagiochila porelloides (Torrey ex Nees) Lindenb.	El Rasillo de Cameros (La Rioja)	1163	42.11	-2.42	*Pinus sylvestris*	4	3	6	6	4	0
Scapania undulata (L.) Dumort.	Lumbreras (La Rioja)	1030	42.08	-2.39	*Pinus sylvestris*	6	3	10	4	2	0
Antitrichia curtipendula (Hedw.) Brid.	Villanueva de Cameros (La Rioja)	1090	42.09	-2.40	*Quercus pyrenaica*	6	3	4	5	2	0
Bartramia pomiformis Hedw.	El Rasillo de Cameros (La Rioja)	1163	42.11	-2.42	*Pinus sylvestris*	4	3	6	4	2	0
Brachythecium rivulare Schimp.	Lumbreras (La Rioja)	1340	42.02	-2.35	*Pinus sylvestris*	6	3	8	6	5	0
Brachythecium rutabulum (Hedw.) Schimp.	El Rasillo de Cameros (La Rioja)	1180	42.12	-2.42	*Pinus sylvestris*	6	x	6	6	6	0
Bryum argenteum Hedw.	Logroño (La Rioja)	370	42.28	-2.25	No forest, urban	8	x	4	6	7	0
Bryum pseudotriquetrum (Hedw.) P.Gaertn. et al.	Lumbreras (La Rioja)	1030	42.08	-2.39	*Pinus sylvestris*	7	x	9	6	3	0
Calliergonella cuspidata (Hedw.) Loeske	Logroño (La Rioja)	370	42.28	-2.25	No forest, urban	8	3	7	7	4	0
Ceratodon purpureus (Hedw.) Brid.	Lumbreras (La Rioja)	1207	42.06	-2.38	*Pinus sylvestris*	8	x	2	5	3	1
Cratoneuron filicinum (Hedw.) Spruce	Nieva de Cameros (La Rioja)	947	42.13	-2.40	Riparian (Salicaceae)	7	x	8	7	5	0
Rhytidiadelphus triquetrus (Hedw.) Warnst.	El Rasillo de Cameros (La Rioja)	1163	42.11	-2.42	*Pinus sylvestris*	7	3	4	5	3	0

The material of each species was separately stored in polyethylene containers with high humidity atmosphere and transported to the laboratory in a portable icebox (temperature below 5°C).

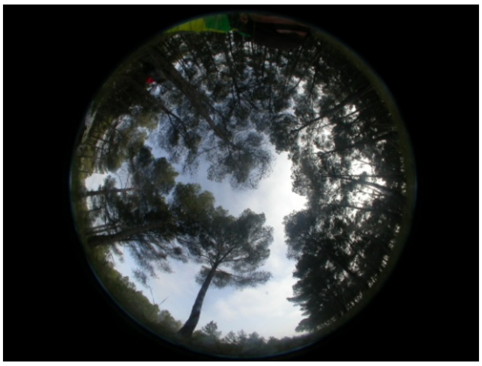

Fig. 2. Canopy photographs of *Hypnum cupressiforme* of the typical semi shade environment.

Maintaining the bryophytes under controlled conditions

Once in UPV/EHU lab, species were transferred to small containers with the substrate (100% turba) and they were maintained at natural densities in a greenhouse for a week before the BVOCs sampling collection and physiological determinations. The conditions of the greenhouse were as follows: 14 hours photoperiod, the average temperature of 24ºC/18ºC day/night, and average humidity of 80/60 % day/night (Fig. 3)

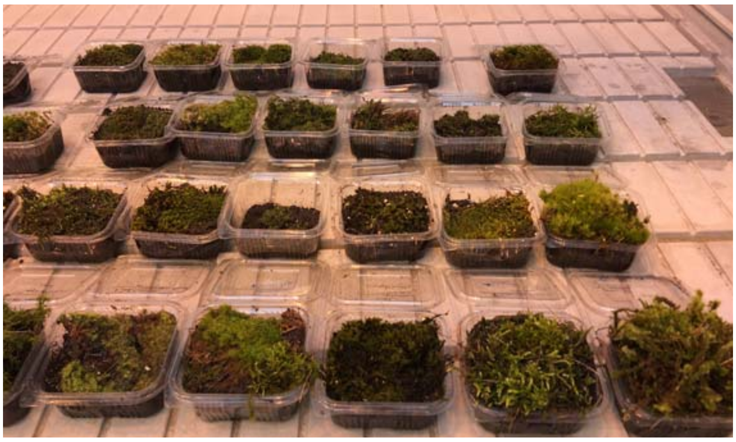

Fig. 3. Bryophytes growing at natural densities at the greenhouses from the Advanced Reseach Facilities at UPV/EHU.

Water content*:* As traits in bryophytes will depend on the hydration status determining the water content is key for biochemical analysis. Standardized tallus densities were sampled and water content was determined in the laboratory by the dry weight (dried at 70 °C for 24 h) and fresh weight determinations.

Leaf-level functional characterization: Maximal photochemical efficiency of photosystem II (Fv/Fm) was used as a proxy of the photosynthetic integrity of the tissue and measured with the imaging fluorimeter (PSI, Czech Republic. It is an automated station, it can record measurements automatically by programming. Fluorescence was detected by a high-sensitivity charge-coupled device camera equipped with an F 4.5–10mm, 1:1.6 objective that produced images in a 12-bit grey scale. Pixel value images of the fluorescence parameters were displayed as a false colour code ranging from green (0.4) to red (0.8). The instrument is driven by the FluorCam software package (FluorCam 6). First, images of the dark-adapted fluorescence level, Fo, were determined using non-actinic measuring flashes. Next, an 800ms duration pulse of saturating light radiation (2000µmol photon m^{-2} s^{-1}) was given. The maximum fluorescence level, Fm, was measured during the saturating light pulse using 12 measuring flashes. The maximal photochemical efficiency of photosystem II was estimated by the ratio Fv/Fm=(Fm−Fo)/Fm (Fig. 4). I also estimated non-photochemical quenching determination, as indicative of plant stress and *in vivo* light curves. Each bryophyte was measured five times with five different replicates.

Leaf chemistry: N and C. Although non-planned in the initial proposal, I have added this tasked to project. The same dried leaf samples employed for water content determinations were ground and the total foliar C and N concentrationare now being measuring with an Elemental Analyzer (Perkin-Elmer CHNS/O 2400, Waltham, USA) after sample combustion in an oxygen atmosphere at 925 C. These measurements are now performed in CEBAS-CSIC facilities with the collaboration of Dr Aranjuelo (CSIC)

BVOCs sampling and volatile composition analysis: bryophytes canopies were exposed to light (200 µmol m^{-2} s^{-1}) before sampling (Tattini *et al.*, 2015). Healthy green apices (1–3 cm depending on the species) were cut and clean from any debris before frozen with liquid nitrogen and stored at -80ºC until further use. The samples were sent to the CNR, Italy where the analysis is now ongoing. This analysis is in collaboration with F. Loreto and M. Michelozzi. The extraction and measurements in the GC-MS were performed as in Catoli et al (Catola *et al.*, 2016) with the modifications established for bryophytes by the applicant.

Pigment analysis: Carotenoids, chlorophylls and tocopherols were extracted and analysed following the method of Fernández et al. (2018) and Esteban & Garcia-Plazaola (2012).

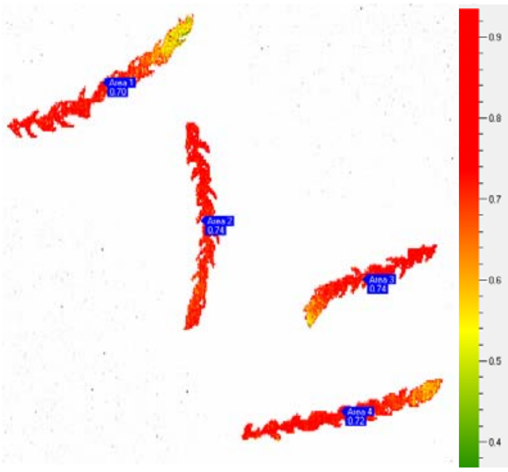

Fig. 4. Photochemical efficiency (Fv/Fm) of *Brachythecium austrosalebrosum* collected the Antarctic. The false colour code depicted at the right of the panel ranges from 0.8 (red) to 0.4 (green).

Microscopy: We collected samples for further analysis by microscopy, in case I have to investigate for BVOCs specific storage structures (Loreto & Schnitzler, 2010) (by fixation with 4% glutaraldehyde / paraformaldehyde 2%). This was not scheduled in the initial proposal.

2.3. Results and objetives fulfilment

I have some preliminary results. However, due to the delay in the samplings and some technical problems with the GC-MS, we still do not have the definitive results. We are now finishing to perform the analysis of the volatiles in collaboration with the CNR, Italy.

Objective 1: To *investigate if bryophytes are important BVOC sources*--**100% completed**

All the bryophytes analysed so far showed a constitutive blend of BVOCs that clearly indicates the importance of these organisms as BVOCs sources. Most common BVOCs found in almost all the species analysed so far were: α-pinene, camphene, limonene, cineolo and terpinolene. A figure with the composition of the liverwort *Porella platyphylla* is depicted (Fig. 5)

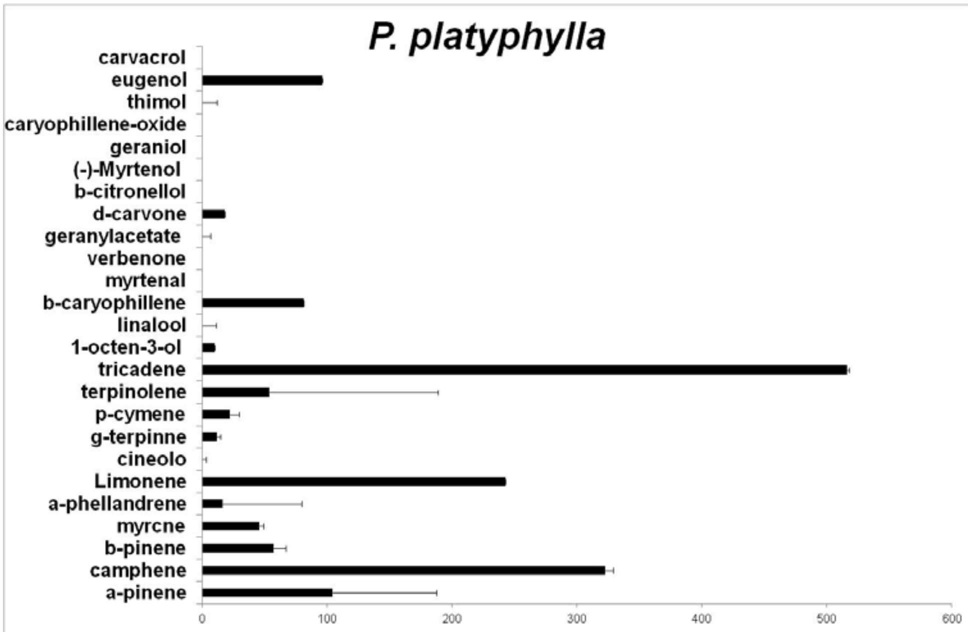

Fig. 5. The composition of BVOCs (ng mg^{-1} FW) in the liverwort *Porella platyphylla*

Objective 2, 3 and 4: To *cover bryophytes BVOC composition among phylogenetic groups and to relate the type of BVOCs emitted with the bryophyte location, habitat or ecological requirements of the species and to discover which is the possible function this emission in bryophytes* --**65% completed**

These objectives are completed by 65% because we still have to finish analysing the BVOCs of the bryophytes and relate the results with the rest of the data. However, I had analysed the photoprotective composition and the physiological characterization of these organisms comparing both biomes (Fig. 6). In general, the constitutive photoprotective composition (total carotenoids and total tocopherols) under non-stressed environment was slightly higher in extreme biomes than in template biomes.

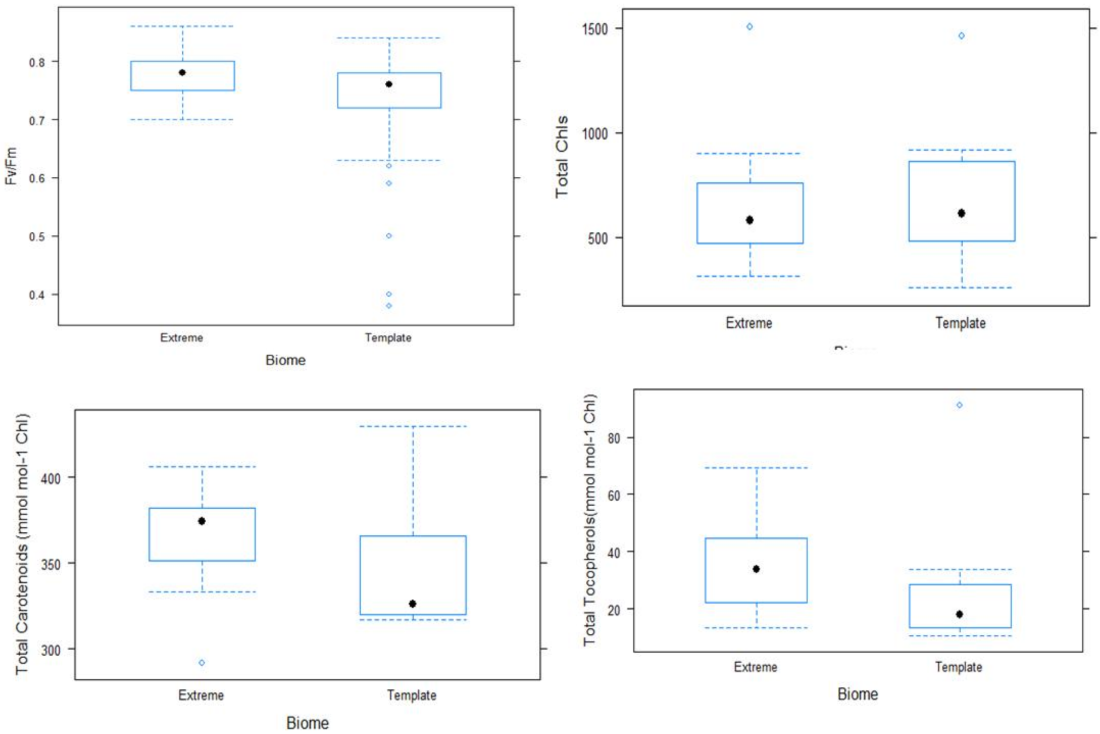

Fig. 6. Boxplots showing the concentrations of the main photosynthetic pigments in bryophytes across biomes: Photochemical efficiency (Fv/Fm), total chlorophyll (Total Chls), Total carotenoids (mmol mol^{-1} Chls) and total tocopherols (mmol mol^{-1} Chl). Central lines represent the medians and whiskers represent the minimum and maximum values.

2.4 Conclusions and evaluation of the execution

This project was really ambitious and it had key risks associated with the four of the objectives (that were most of them previously contemplated in a contingency plan). In this sense, the careful planning and reassessment of the risks during the execution of VolNaBry mitigates the risks, however, it delayed the fulfilment of the project.

The sampling was more complicated than previously planned, and two of the four locations failed for the initial plan: Nicaragua and Finland (due to political problems in the country and to collaborators withdrawn, respectively). Due to the contingency plan, additional two sites sampling were immediately planned to substitute Nicaragua by Vietnam, and Finland by Minnesota. To mitigate this problem, I also add new sites that were not previously contemplated, Chile and The Antarctic.

Another risk was related to metabolomics. It was a challenge to determine BVOCs in non-model species, as bryophytes (high variability among metabolite profiles within treatment groups, a high number of "unknown compounds"...). I lowered the impact of this risk by the standardization of metabolites acquisition and modifying the extraction protocols myself.

We are now performing all the BVOCs analysis, so probably by the end of February, we will have all the definitive results. We found that bryophytes are important BVOCs sources, which is essential towards greater scientific integration of BVOCs understanding. With this work, a BVOCs blend trait database in bryophytes is now been set up, making possible to explore in this group the relationships between emissions and ecological characteristics. As there may exist large differences in BVOC emission blend when comparing extreme/different biomes, this work will clarify the interaction between ecological strategies and emissions and will discern relationships between emissions and the environment.

Although this project was not completely fulfilled, I strongly think that VolNaBry was of great success and a high-gain project. Indeed, this project was the seed for the germination of a new line of research that will yield many important outcomes (see resulting publications section).

2.5. Resulting publications

The VolNaBry project has resulted in the following oral communication: Esteban R, Pollastri S, Brilli F, García-Plazaola JI, Martínez-Abaigar J, Nuñez-Olivera E,Tattini M, Michelozzi M, Loreto F. El mundo volátil de los briofitos". XIII Coloquio Primavera Ecofisiología, Abril 2018, Cercedilla, Madrid, España. I had also explained my results and the project in a conference inside the master "Agrobiotecnología ambiental" at the UPV/EHU. I will also present the final results of the project in the coming SEFV conference (https://www.fv2019.org/modules.php?name=webstructure&idwebstructure=11). Besides, the results of VolNaBry have been the based (the seed) for applying for the prestigious ERC starting grant project: Traits and ECo-chemicals LInk for non-vascular and vascular PlantS: prediction at the plant- and ecosystem-level (ECLIPSE), which is actually under evaluation. A manuscript is also under preparation for sending at the end of 2019.

Budget

Raquel Esteban, as the beneficiary of the present project from the call 2018 8th Ed. "Proyectos de investigación liderados por jóvenes investigadores", once finished the data collection, I present here the economic budget. The vast majority of the budget has been spent in the BVOCs analysis (77%), as it was contemplated in the initial proposal. The rest of the budget was used for growing containers and substrates for bryophytes (1%), sampling expenses (15%), sample sending (10%). I also bought a fisheye for ambient characterization for completing the characterization of the bryophytes environment at the time of sampling. It is important to highlight that I expend 172 € more than the budget. In the following table, a summary of the budget is depicted. I also attached the scanned invoices, according to the column "code" of the table.

Concept	Date	Amount (€)	Invoice code
Fisheye for ambient characterization	20/03/2018	49.99	1
Sampling and growing containers	26/03/2018	14.00	2
Sampling at la Rioja. 310 km (*0,19€)+	24/04/2018	58.90	3
Toll	24/04/2018	29.05	3
Substrates	03/05/2018	18.40	4
Samples sending from La Rioja	30/02/2018	30.82	5
Vietnam sampling	03/10/2018	377.74	6
BVOCs analysis	17/09/2018	2330.00	7
Samples sending to Italy	26/09/2018	263.91	8
Total (€)		3172.86	

Acknowledge

The IP of this project would like to thank all the collaborators implicated in VolNaBry. I would like to especially acknowledge Javier Martinez Abaigar, the group Ecofisco of the University of Basque Country (particularly to M. López and U. Artetxe). Finally, I much appreciate this outstanding opportunity provided by the AEET.

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