





# User's guide for Ecofog's Ecophysiology Lab

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# Introduction

Plant functional traits are the features (morphological, physiological, phenological) that represent ecological strategies and determine *describe?* how plants respond to environmental factors, affect other trophic levels and influence ecosystem properties. Variation in plant functional traits, and trait syndromes, has proven useful for tackling many important ecological questions at a range of scales, giving rise to a demand for standardized ways to measure ecologically meaningful plant traits. The importance of these topics dictates the urgent need for more and better data, and increases the value of standardized protocols for quantifying trait variation of different species, in particular for traits with power to predict plant- and ecosystem-level processes, and for traits that can be measured relatively easily (Pérez-Harguindeguy et al., 2013)

This handbook presents the different protocols used in the ecophysio lab. We therefore suggest the methodological principles for a more open and transparent science. This handbook not only includes updated methods for the trait measurements, but also includes the excel worksheets for data collection and links toward detailed tutorial or user's guide for all methods used in this lab. This handbook will be associated with a package `EcophyCofog` containing some usefull homemade functions to deal with some devices output.



# Handbook architecture

This handbook is written for operational ends. As such, it is not a review or scientific paper thoroughly presenting each traits but rather a list of protocols associated with routinely measured traits in this lab.

Each chapter of this book correspond to one trait and associated measurement process.

Morpho-anatomy

Hydraulics

Fluorescence

Gaz fluxes and exchanges

Microbiology and metabarcoding

Greenhouse setups and tips

Device info



# Morpho-anatomy

## 2.1 Shoot traits

Shoot traits refer to all morpho-anatomical traits that can be measured on plant aerial part. Most of these traits are measured on the leaves but see [Stem](#) section.

### Mass associated traits

Plant growth strategies and overall performances are often assessed via mass associated traits. Three masses are used to calculate different metrics. The *Fresh Mass (FM)* which is the weight of freshly sampled leaves. The *Turgid Mass (TM)* which is the mass of fully hydrated leaves. Leaf hydratation protocols are source of debate in EcoFog but are presented [here][Leaf hydratation] And the *Dry Mass (DM)* which is the mass of dried leaves.

To be compared, these weights must come from the same sample and we must measure the leaf projected surfaces. See [here](#) for the leaf surface measurements protocols.

From these masses and surfaces are calculated metrics such as SLA/LMA, RWC, LDMC, WHC ([Calculation](#)).

**Use the same balance for all your masses within an experiment!!**

### Leaf surface

Most of leaf surfaces are obtained from leaf scans using office scanner. The used scanners are [EPSON's V800](#). To acquire leaf scans please see [Image Acquisition](#). Depending on your research question you can either scan one leaf or several leaves (to have total leaf surface of your plant for instance).

## 2. MORPHO-ANATOMY

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Once you have one scan per experimental unit you need to extract its surface. Surface measurements are made with the [ImageJ](#) freeware. [ImageJ](#) works on all OS and can be downloaded [here][\[https://imagej.nih.gov/ij/download.html\]](https://imagej.nih.gov/ij/download.html)

Surface can be measured **manually** or **automatically**

**Manual measurements :**

Launch [imageJ](#) -> File -> Open (Ctrl + O) and browse for the picture you want to analyse.

Then select the **Polygon selection tool** [2.1](#).

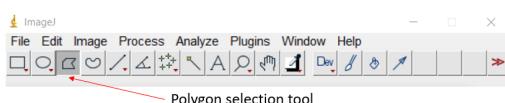


Figure 2.1: Polygon selection tool in ImageJ

Zoom (-/+ or **Ctrl+wheel in/out**) into your image to see only the leaf you want to measure [2.2](#).

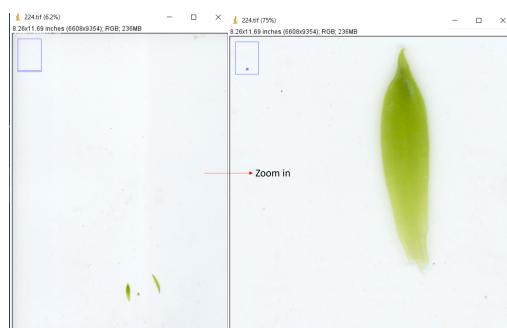


Figure 2.2: Zooming in ImageJ

Then you can **left click** to detour your leaf [2.3](#). The more points you have the more precise and close to the leaf your measurements will be. However, bear in mind that this is laborious and that as you zoomed in your error should be negligible.

Once you've closed your polygon, press **ctrl+M**. A window containing the results will pop-up [2.4](#).

The Area will be expressed in the units of your photo. Some pictures have embedded scales in cm or inches but the default is usually in pixels (px). For more information see [\[ImageJ scale\]](#).

Each measurements will be added to the results window (one each time you press **ctrl+M**). You can then save this window in the .csvformat clicking on **File**, **Save As** and browse for chosen location. You can either have one file per photo (i.e. sample) and then name your file as your sample ID. Or you can decide to have one file for all your samples. Then you have



Figure 2.3: Leaf detouring in ImageJ

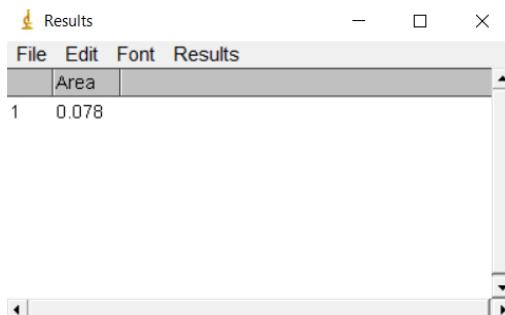


Figure 2.4: Results window in ImageJ

to remember or write somewhere which measurements ID is associated to each of your samples to find who's who 2.5.

#### Automatic measurements:

This will allow you to analyze many data at the same time. To do so you will need to use an homemade ImageJ macro.

**Macro exemple.** This macro is used to convert your colored scans in 8 bit black and white pictures. Then it set scale for 800 ppi scans (distances=800px known = 2.54 cm , pixel = 1). With ppi -> pixel per inch and 1 inch = 2.54cm. The pixel ratio is 1 as vertical and horizontal resolution is the same. It sets the contrast and then anaylze size of particle bigger than 0.01 cm<sup>2</sup> and smaller than infinity. It displays the results in summarize form for each pictures in the folder.

See [here](#) for a tutorial on macro recording in imageJ.

## 2. MORPHO-ANATOMY

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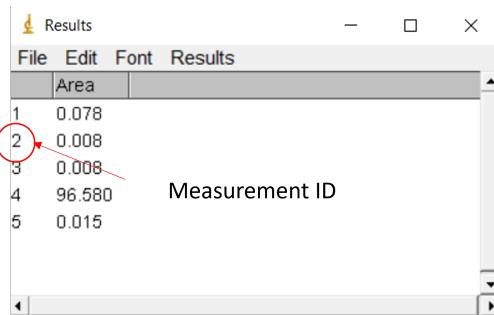


Figure 2.5: Measurement ID in ImageJ

Table 2.1: Mass derived traits

Acronym	Full name	Formula
LA	Leaf area	
FM	Fresh mass	
TM	Turgid mass	
DM	Dry mass	
LMA	Leaf mass area	$DM/LA$
SLA	Specific leaf area	$LA/DM$
LDMC	Leaf dry mass content	$DM/FM$
LS	Leaf succulence	$(FM - DM)/LA$
RWC	Relative water content	$[(FM - DM)/(TM - DM)] * 100$
WHC	Water holding capacity	$(TM - DM)/LA$

To launch a batch process go to **Process-> Batch-> Macro....** This will open a window. Fill the fields as follow: + **Input:** folder containing your pictures to be analyzed + **Output:** folder to receive your output. + **Output format:** quite clear!

### Calculation

### Leaf anatomy

#### Stomata and trichomes density

Stomata and trichomes density are acquired via epidermis printing. First, apply double sided tape on your leaf and remove it. You now have a piece of tape with your trichomes on it. Place it on a glass-slide with the trichome-covered side on the top.

Then, to get your stomata, apply a thin coat of transparent nail polish and let it to dry. Then you remove it with double-sided tape and put it on a glass slide (nail polish on top).

Observations are made with the [Olympus BX51](#) microscope.

And pictures are taken with the installed camera (currently [Lumenera LW1135C-IO](#) but about to change).

Pictures can then be uploaded in ImageJ and you can manually count stomata and trichomes using the `multipoint` tool.

There is a new opportunity to use `LabelStoma`, a deep learning software for stomata detection. It is developed by Ángela Casado-García and we are trying to make it work on some species that we study at EcoFog.

### Anatomical structure

Leaf anatomical observations are made on razor blade hand-made cross sections of leaves.



Figure 2.6: Hand made cross section of a bromeliad leaf

These cuts are directly placed in water/oil on a glass-slide and observed with the [Olympus BX51](#) microscope.

And pictures are taken with the installed camera (currently [Lumenera LW1135C-IO](#) but about to change).

**Always use the same picture format!!** We recommend JPEG or TIFF.

On these pictures you can then make measurements such as width of the epidermis and cuticle. The width of certain type of tissue (chlorenchyma, parenchyma, hydrenchyma) or the distance between vessels using ImageJ tools such as the `draw segment` and `ctrl+M` output (area, angle of the line and length).

**SEE MATTHIEU PICS**

## Stem

## 2.2 Root traits

Root morphology analysis (length, diameter, etc.) are conducted using the Winrhizo software.

## 2. MORPHO-ANATOMY

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Winrhizo is a licenced software created by Regent Instrument Canada Inc. It exist 4 different version and we own the *Basic Version*. It allows root morphology analysis from scans.

### Image Acquisition

#### Format

Supported image format are .TIFF, .JPEG and .BMP. .TIFFand .BMP are not compressed and are thus to be preferred. .TIFF images are compatible with all OS and should be privileged but you must be careful to save them *uncompressed* as WinRhizo won't be able to open *compressed* ones.

The higher the resolution, the more pixel you will have and the more precise will be your measurements. However, with resolution, scan time and image size increase. 800DPI is the standard in this lab but 400 is the winrhizo recommendation. This depend on the required level of precision as well as the size of the analyzed roots ( the finer the higher must be the resolution to get more details).

#### Scanner

Any scanner can be used to acquire scans for Winrhizo software. However, be sure that the format is compatible and that all the images inside your project are saved in the same format and the same resolution. For coherence purposes we encourage you to use the same formats between studies at Ecofog's lab scale. [EPSON's V800](#) scanners are the ones used as this document is being written. The scanner model isn't important but we recommend to use scanners with a transparent (double-lamp) option. This will allow cleaner root scans for complex root systems. And the scanning software is [ViewScan](#)

#### Scan process

Paste the wall-taped doc.

**Flat scan** You can decide to use basic scan options with light only coming from below. If you do so you need to have a white background installed under the scanner's roof (if black roots, if pale ones you'll need a black background).

Choosing this option will simplify your protocol and can suffice for simple and thin enough root systems.

#### EXAMPLE scan marion

**Transparent** If your root are too big [2.7](#), then self-shading can appear on flat scan and bias winrhizo's analysis. To avoid this shading you can remove the background from the scanner's roof to enable double-lamp scanning.

The light coming from top and bottom as one, shading will be avoid and scans will be cleaner.



Figure 2.7: Roots too thick to be flat scanned

Another case where you can prefer **Transparent** option is for complex root systems (e.g. bromeliaceae, [2.8](#)) . For this type of roots, you can scan them in a thin coat of water to disentangle fine roots. Doing so you will have a better analysis of the root system morphology and structure but once again have shading issue. Supressing them requires the use of the **Transparent** mode.



Figure 2.8: Complex bromeliad adventitious root system

**BEWARE:** The **Transparent** scan window is smaller than the normal mode scan. The actual scanned zone is showed [2.9](#) and you must make sure that your roots are well placed within this area.

## Image processing

To analyze with winrhizo, you can either make it manually, one image at a time and by drawing rectangles around the roots you want to analyze. However, when having a lot of scans you might want to automatize the process using the **batch** option. If this is your choice, make sure that your images only contain roots!! Sometimes you will have to remove some parts of the scans to leave only roots in your images. For instance, this [2.10](#) is the

## 2. MORPHO-ANATOMY

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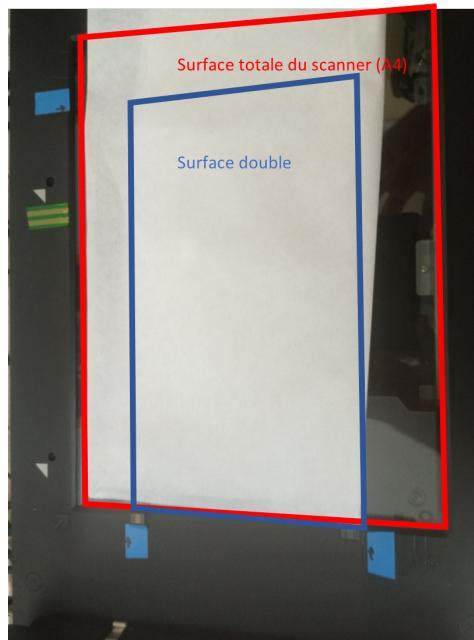


Figure 2.9: Flat (red) and Transparent (blue) scan zone of EPSON's V800 scanner

scan from bromeliads roots. We can see the water-filled petri dishes border on the scan and this will be an issue for automatized Winrhizo analysis.



Figure 2.10: Scan of a bromeliad root system in water-filled petri dish

To re-crop these images we use the freeware XnConvert. The petri dish has always been placed in the same place using a stencil ?? on the scan window, enabling us to recrop all scans to the same size. Detailed XnConvert tutorial is available [HERE](#).



Figure 2.11: Stencil used for inwater root scans



Figure 2.12: Stencil used for inwater root scans

## WinRhizo

### Installation

The winrhizo software is contained on a CD (ask [Eliane Louisanna](#)). To be used you need to copy the software from the disk to your computer and install the protection key drivers (also on the CD). Once installed you don't need the CD to run the software but the protection key must be plugged. Unplugging it will prevent any use of the software.

### Startup

#### First analysis

Once you have acquired your images and launched winrhizo you can start to analyze your scans. To display a single scan, click *Image -> Origin -> From File*. Then you can click the *acquisition* icon **PIC**. This will open a standard document opening window. Then you browse normally to find the wanted scan. Make sure that you are looking for the good format, by default, winrhizo display .TIFF. When you open it, winrhizo display the targeted image and you can then click on it (analyze whole image) or make a selection (only selected region) to start an analysis.

When an image or region is analyzed, winrhizo display the *sample identification* window which allows you to enter information about the sample. These informations will be saved with the measurements data. In this window click *OK* to do the analysis or *Cancel* to abort it.

After you clicked *OK*, winrhizo starts the analysis (can be stopped pressing *S*). When done, winrhizo is ready to save the data but a file must be opened or created first. Winrhizo display a window which asks whether to *open one*, *create one* or *save nothing*. Selecting *create one* will create a new .TXT file to store analysis data (more info about output [here](#)). Selecting *open one* will allow you to open a pre-existing file to add the new measurements at the end of this file. Clicking on *save nothing*, guess?

In the image, you can now see which roots have been analyzed. Those have a skeleton line over them. The absence of this skeleton indicates that these roots have not been analyzed. This can be due to non-optimal pixel classification (see more about it [here](#)).

WELL DONE! You just analyze your first picture!

You can practice with scans available [here](#)

### Calibration

If not calibrated (associated with a scale), winrhizo will display results in pixels. .TIFF files have an embedded scale, automatically detected by winrhizo. Check on your results if they are in px (pixels), in (inches) or cm (centimeters).

However, you can sometimes have uncalibrated files (mistakes or images from camera). Thus, you will need to “manually” calibrate your image. Winrhizo calibration files are saved as .CAL. In the *Calibration* menu you can load pre-existing calibration files. You will find the `calib_imge.TIFF` [here](#). To make your calibration at any DPI, you can print this image and scan it at the wanted DPI. The black square in the image delimit a white 1x1cm square. Loading this image in winrhizo, you can click on *calibration -> pixel size method -> object of known dimension -> 1 image -> width=1, height=1, border=0.35, units=cm -> Ok*

Then, winrhizo will propose you to save the calibration in a .CAL file that can be loaded later and used for all your images at a given resolution. **DO NOT NAME YOUR FILE Scanner.cal**. Please, when you create a .CAL at a previously not used resolution, store a copy of the calibration file [here](#) so that your work helps your successors!!

### Batch

We saw how to analyze a [single picture or region](#) but you might have numerous scans to analyze and want to automatize this process. To do so you will give winrhizo a *batch* (i.e. a folder) containing any number of images you want.

### Pixel classification

### Output

## 2.3 Resources



# Hydraulics

## 3.1 Leaf turgor loss point, $\pi_{tlp}$

We assessed the leaf turgor loss point,  $\pi_{tlp}$  in MPa, from a previously established relationship with the osmotic potential at full hydration,  $\pi_{osm}$  in MPa.  $\pi_{osm}$  is linked to the equilibrium solute concentration value  $C_0$  (in mmol.kg<sup>-1</sup>) directly measured with a vapor pressure osmometer (Vapro 5600, Wescor, Logan, UT). This is referred as the *osmometer method* (Bartlett et al. 2012a; Maréchaux et al. 2016).

### Materials

- Vapor pressure osmometer (Vapro 5520, Wescor, Logan, UT)
- Vapro software (Vapro Lab Report)
- Fridge
- Liquid Nitrogen
- Ziplock bag
- Paper towel
- Distilled water
- Metal tea ball
- Tin foil
- Needle
- Liquid nitrogen gloves + goggles
- Liquid nitrogen contenant
- 2 Tweezers
- Cork borer

### 3. HYDRAULICS

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## Methods

### Installing Vapro for measurements

- Turn on Vapro the day before for the thermocouple's stability
- Test Water Quality *cf Vapro\_cheatsheet*
- Clean
- Calibration *cf Vapro\_cheatsheet*
- Control tests *cf Vapro\_cheatsheet*
- Verify temperature
- Always have the black diamond at the center of the display

Used daily: \* clean beforehand \* select automatic mode (10 runs)

### Sampling on the field

- Collect at least 3 healthy mature leaves on branch
- Place them in sample ziplock bag with:
  - wet paper towel
  - Exhale in bag to saturate in CO<sub>2</sub>
  - Annotate bag with sample information
- Zip bag and stock in cooler

### Lab measurements

#### Field day

- Recut branch under water
- Replace in ziplock bag with wet paper towel
- Put 24h in fridge to hydrate overnight

#### N+1 Field day Vapro:

- check distilled water in vapro reservoir
- clean
- select automatic mode (10 runs)
- make sure vapro software is on

Sample measurement:

- Sample from a leaf a 5 mm disc with a cork borer: *avoid 1<sup>st</sup> and 2<sup>nd</sup> order veins to avoid apoplastic dilution that would lead to less negative osmometer values*
- Wrap disc in tin foil
- Immerse in liquid nitrogen for at least 2 min using metal tea ball
- Puncture 10-15 times with needle

- Place in vapro chamber

In total, disc are exposed to air for less than 40 seconds for all the steps.

- Record value  $C_0$  when the difference between consecutive 2-min measurements fell below strictly  $5 \text{ mmol.kg}^{-1}$  after at least three runs.
- If error! or Nr\_Run > 10 :
  - try a 2<sup>nd</sup> cycle with same leaf
  - try a 3<sup>rd</sup> cycle with another leaf
  - otherwise record NA
- Beware of the stuck leaf inside the vapro! If so cf *Vapro\_cheatsheet*

**End measurements** Clean Vapro

For more information on the vapro machine, please refer to the *vapro cheatsheet* in the machine category.

## 3.2 Leaf midday water potential

**Pressure chamber method**

**Psychrometry method**

## 3.3 Relative water content RWC (%)

### Material

*Ziplock bag* Paper towel *Distilled water* Fridge *Analytical balance* Sharpie  
*cooler envelop*

### Method

Before going on the field: \* write individual code on ziplock bag \* preweight ziplock bag

On the field: \* collect leaf \* clean leaf with clean paper towel \* place leaf in preweighted corresponding ziplock bag \* place bag in cooler for transport to the lab

In the lab: \* weight the closed ziplock with the leaf (*fresh weight*) \* delicately take the leaf out of the bag, wrap it in moist paper towel and place the wrap back in the ziplock bag \* place ziplock bag in the fridge during 24 hours \* 24 hours later, take out the leaf and wipe off water excess \* weight re-hydrated leaf (*saturated weight*) \* place leaf in envelop for oven-drying during at least 72h \* weight dry leaf (*dry weight*)

### 3. HYDRAULICS

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Calculate RWC (%):

$$\text{RWC} = \frac{(\text{fresh weight} - \text{dry weight})}{(\text{saturated weight} - \text{dry weight})} \times 100$$

From Sapes et al 2020 and Barrs & Weatherley 1962.

#### **3.4 gmin**

#### **3.5 gs**

# Fluorescence

Fluorescence measurements are made with the [MiniPAM II](#) fluorometer. It is used to assess the efficiency of plant photosystems to convert light into chemical energy.

[Maxwell and Johnson 2000](#) provided a good synthesis of the theories behind these measurements.

## 4.1 Theory

We will try to summarize it a bit here. Light energy is either converted into Photochemical energy, fluorescence or heat. Measuring fluorescence can give information about the other if we control one (i.e. photochemistry).

We use the Kautsky effect, a variation of the fluorescence pattern when exposing the leaf to light. When passing from darkness to light, photon will saturate the plant photosystem II and  $e^-$ - acceptors. The PSII are said “closed” until  $e^-$  go down the reaction chain.

Thus, for a few seconds, the system cannot do photochemistry and only emits fluo (measurable) and heat!!!

After this period, the fluorescence starts to fall again ==> fluorescence quenching

Because :

1 -  $e^-$ - transfer away from the PSII increases with light activated enzymes. : **photochemical quenching**

**2 - Heat energy estimation : Non-photochemical quenching** 15  
to 20 min are needed to reach the equilibrium but it varies between species.

#### 4. FLUORESCENCE

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For usefull informations, we need to supress one of the 2 (typically 1). Indeed, if no photochemical quenching (PQ), then we only have non-photochemical quenching (NPQ).

We thus use the **light doubling technique**.

An high intensity-short duration flash closes all PSII.

During this flash, the fluorescence yield reach values attained with no NPQ ( $F_m$ ).

$F_0$  is the fluorescence level when no actinic light (darkening + far red to open reaction centers).

$F_t$  is the steady state of fluorescence in actinic light.

NPQ can too change with time and this is reflected as  $F_m$  changes when PQ is off/negligible.

We can then calculate :

$\phi_{SII} = \frac{F'_m - F_t}{F'_m}$  which is the proportion of light absorbed by PSII chlorophyll and used for photochemistry.

and :

$qP = \frac{F'_m - F_t}{F'_m - F'_0}$  the proportion of closed PSII reaction center, it increases with plant stress.

$F_v/F_m = \frac{\phi_{PSII}}{qP} = \frac{F'_m - F'_0}{F'_m}$  or with  $F^{\circ}_m$  and  $F^{\circ}_0$  in the dark adapted state.

## 4.2 Fv.Fm

To measure Fv/Fm with the MiniPAM you have to use the dark leaf clip and place one on the target leaf for 30 minutes dark adaptation. Then press record on the MiniPAM screen to create a new data entry. And, finally just insert the optical fiber in the leaf clip, open it and press Fv/Fm on the MiniPAM screen. You can either note the displayed value manually or extract it later with the MiniPAM associated software [Wincontrol](#). If you chose the later, do note the correspondance between your subject ID and the record created by the MiniPAM.

C H A P T E R



## Gaz fluxes and exchanges



C H A P T E R

# 6

## Microbiology and metabarcoding



C H A P T E R

7

## Greenhouse setups and tips



# Device info

## 8.1 Vapor pressure osmometer - Vapro 5520 cheatsheet

- $\pi_{TLP}$  vapro cheatsheet

This template is based on *Bookdown* and the *Memoir* LaTeX class to allow writing a book, a report, a PhD thesis, etc. in *R Markdown*.

The main file is *index.Rmd* which contains the description of the book in its header. All other *.Rmd* files in the folder contain a chapter. The *references.bib* file contains the bibliography.

This file will have to be deleted, as well as *81-getting\_started.Rmd* and *82-syntax.Rmd*: they have to be replaced by the content of the book.

To get started, create a new R project from this folder. Then open *index.Rmd* and click on the *Build Book* button in the *Build* window of Rstudio.



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**Abstract** English abstract, on the last page.

This is the user's guide of EcoFoG's ecophysiology lab

**Keywords** Keyword in English, As a list.

