Explicet Handbook

"Explicet" is from the Latin: explain, unfold, extend, set forth, exhibit, disentangle

Graphical user interface software for metadata-driven
management, analysis, and visualization of microbial ecology data

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Introduction and Overview

I.   Synopsis

Explicet is a visualization software application that facilitates analysis of taxonomy-based microbiome datasets. The goal of the Explicet development process was to create a powerful, yet intuitive, software tool for exploring and displaying complex microbiome datasets. Utilization of a graphical user interface (GUI) allows the user to concentrate on the science of a microbiome project, rather than the computational details.

Explicet is multi-platform and is currently provided for Mac OS X (10.6, 10.7, 10.8), Windows (XP, Vista, 7, 8), and Linux (RHEL/CENTOS 5, 6). Explicet is fully compatible with taxonomically classified microbiome datasets produced by bundled tools such as Qiime [1], Mothur [10], and XplorSeq [4].

Explicet uses taxonomic classifications derived from rRNA sequence data, combined with complete integration of project metadata, to produce tables and graphical plots (such as x-y charts, heatmaps, pie charts, and stacked bar charts) that portray the make-up of microbial communities from many perspectives. Explicet includes the ability to calculate and graphically display statistical metrics of alpha and beta diversity such as Sobs, Chao1, Good's coverage, and Morisita-Horn. Additionally, Explicet includes several statistical computations (Two-Part, Two-Proportions, and Wilcoxon) that measure the statistical association between a given taxonomic classification type and a categorical variable (e.g., "healthy" and "sick"). Explicet provides data management capabilities that allow a dataset to be subdivided by metadata, sample name, and/or taxonomy. All tables and graphics generated by Explicet may be saved as named "figures" that can be recalled for modification.

Graphics output by Explicet may be in either bit-map formats (such as .png) or vector formats (.pdf), which are readily imported by tools such as Microsoft Word, PowerPoint, and Adobe Illustrator. Tables generated by Explicet are formatted as comma-separated-value files (.csv), which can be easily imported into Microsoft Excel, GraphPad, R, and SAS.

Although developed to facilitate the analysis of rRNA sequence data, Explicet can also be used to analyze other count-based datasets with hierarchical structure (e.g., metagenomic data, gene sequences other than rRNA).

II.   Why Use Explicet?

By their very nature, microbiome datasets have many sub-components; often they contain data derived from many samples, with many sequences comprising each sample, many results derived from each sequence, and all associated with metadata that describe the nature of the samples and the hypothesized sources of variability in the results derived from the sequences (Figure 1).
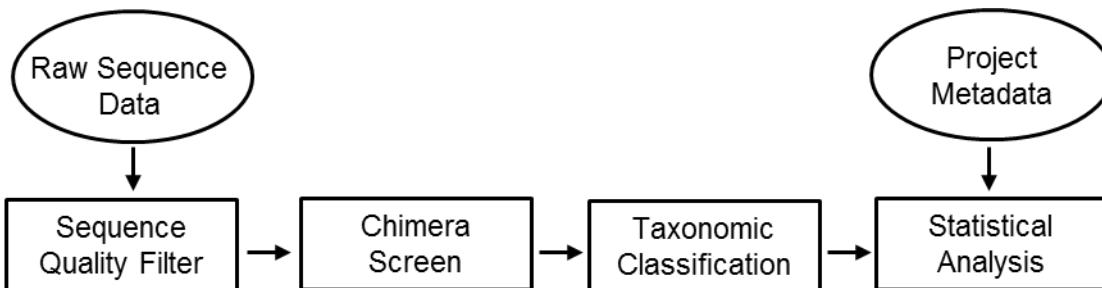
Figure 1. Simplified SSU rRNA Microbiome Data analysis flow

The manual, human-intensive effort required to manage the logistics of these complex datasets often significantly distracts from the scientific questions that prompted the study in the first place. Although microbiome analysis tool frameworks like Qiime and Mothur have made it more tractable to transform raw sequencer data objects to data that can be operated on by statistical tools like SAS [9] or R [8], a significant burden is still placed on the user, because current generation tools are principally command line driven and give little intrinsic data management support. Explicet was developed to ease the analysis of taxonomically classified microbiome datasets by providing metadata-driven data analysis and management capabilities from within a familiar and intuitive GUI. Explicet's capabilities pick up at the point in the data analysis process at which sequence data have been transformed (by means of the user's preferred sequence analysis pipeline) to taxonomic classifications. Once a project's taxonomic and metadata components have been imported, Explicet can be used to subdivide the data by sample, metadata item value, and/or taxonomic attributes, and then apply a variety of analyses to the data. Results can be displayed as tables or graphics (x-y plot, heatmap, pie chart, stacked bar chart, etc.) or can be exported in formats suitable for manipulation by dedicated statistical packages (e.g., SAS, R, SPSS). Each project subdivision of interest can be stored in a separate workspace, all of which is contained in a single project file. Each of the graphics can be saved as "figures" which are easily recalled from the top level of each project.

III. Terminology

A. Descriptive Terms

OTU: An "Operational Taxonomic Unit" is a collection of related sequences that represent an organism or group of organisms. In other words, the sequences define an OTU, the latter of which we believe to map onto a group of organisms. Explicet views numerical OTUs as interchangeable with classification OTUs.

Metadata: Information about a collection of data (e.g., sample type, GPS coordinate, pH, patient, disease status, age, weight, etc.).

Library: A set of OTUs derived from a discrete sample/specimen.

B. Ecological Terms

Biodiversity: The degree of variation of life forms within a given ecosystem or biome. Biodiversity often is measured by the following indices:

Richness: The number of different OTUs in a given environment.
Q: How diverse is the sample?

Complexity: Diversity indices like Shannon and Simpson are attempts to quantify the number of types of organisms present while simultaneously measuring how all of the organisms in a sample are distributed among the types (the evenness of the sample).

Evenness: Quantifies how equally distributed are the OTUs in a library.
Q: How evenly distributed are the members of the community?
Q: Is there one dominant species, or are all species seen in the same abundance?

Alpha Diversity: The biodiversity within a particular sample; usually expressed as species richness.

Rarefaction: Process that randomly resamples all libraries to the sampling depth of the smallest library (called the rarefaction point), thus allowing the alpha diversity metrics to be compared directly.

Beta Diversity: The degree of overlap in biodiversity between two environments, or samples; involves comparing the number of taxa that are shared by two libraries.

IV.   Data Management Capabilities in Explicet

The fundamental data item in Explicet is the "project", the data for which is stored in a single file. Optionally, a project may have multiple derivations called "workspaces" contained within the same file, which typically are subsets of the data contained in the full project. A project and its workspaces may have multiple figures (graphical or tabular representations of data). Projects have direct access to all figures contained within each workspace.
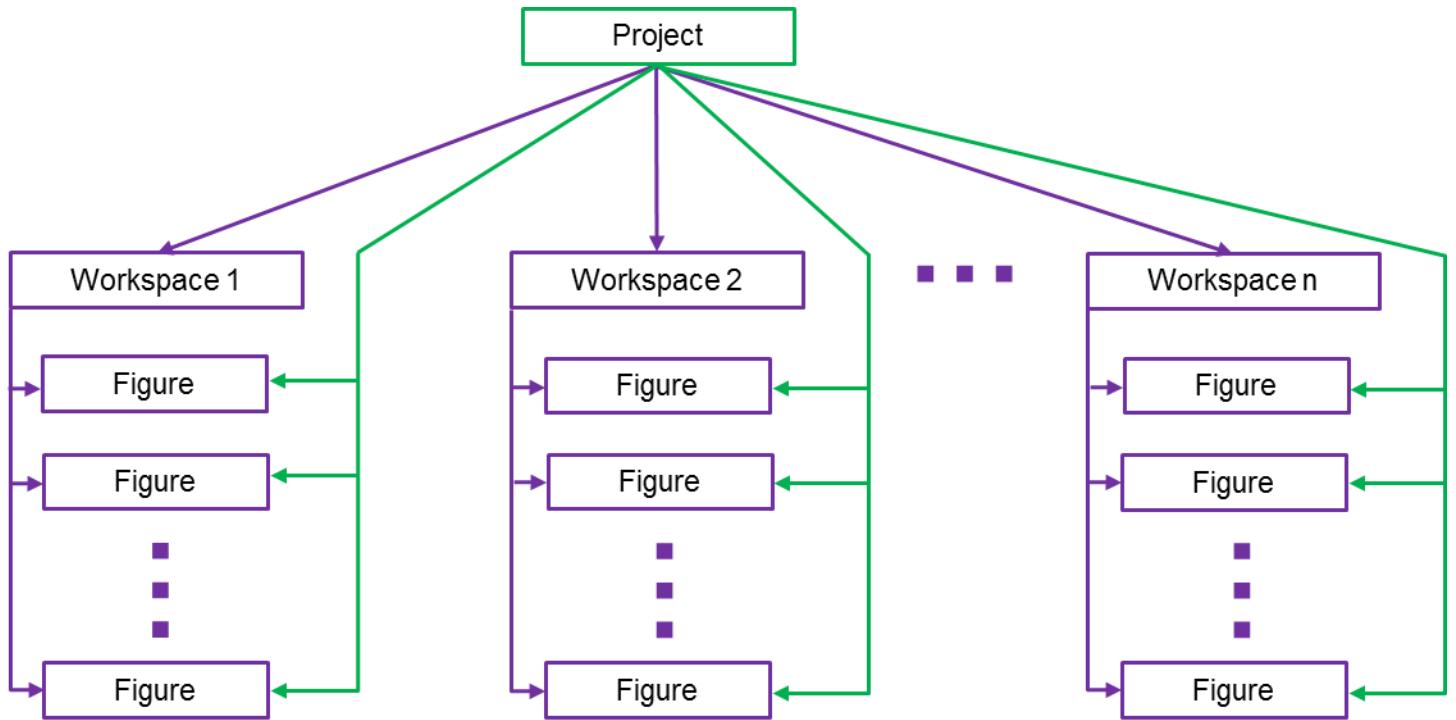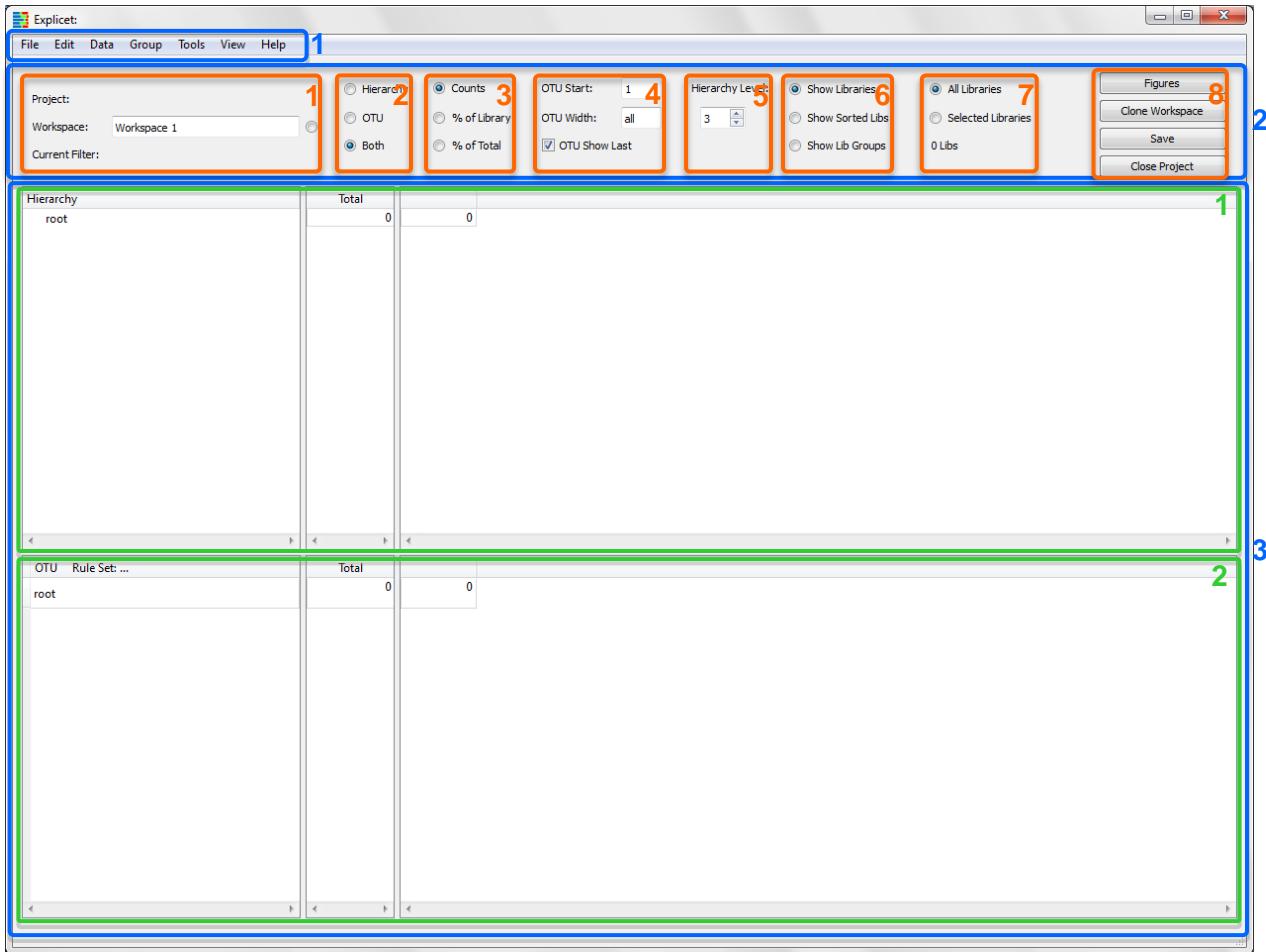
Figure 2. Data management capabilities of Explicet

V.   Main Window Overview

The main window of Explicet has three parts. These are, from top to bottom:
1. The classic graphical user interface pull-down menus consisting of **File**, **Edit**, **Data**, **Group**, **Tools**, **View**, and **Help**.
2. The command and control cluster, used to display and select the options in effect for a project and workspace.
3. One or two data display panes.

The command and control cluster is organized into 8 columns (from left to right):
1. The **Project** / **Workspace** / **Current Filter** column displays the name of the dataset, the name of the current workspace, and the name of a filter that can be used to select libraries within that workspace. Note that the workspace line ends with a radio button that, if clicked, converts the text box into a drop down menu that allows the user to open or move to any workspace within the project.
2. Selects which data display panes (described below) are displayed: **Hierarchy**, **OTU**, or **Both**.
3. Selects how the data is displayed within the **Hierarchy** and **OTU** panes.   Data can be displayed as **Counts**, **% of Library** (a.k.a. percent abundance), and **% of Total**.
4. Allows the user to interactively control the detail in which OTU names are displayed within the OTU pane. **OTU Start** selects the first taxonomy category (counting from 1) to be shown. **OTU Width** determines the maximum number of categories shown. The check box, **OTU Show Last**, can optionally force the terminal category on an OTU name to always be shown independent of the state of **OTU Start** and **OTU Width**. Setting **OTU Width** to zero, and checking **OTU Show Last**, causes display of only the terminal category for each OTU name.
5. **Hierarchy Level** controls the default number of levels that are expanded in the Hierarchy column of the Hierarchy pane.

6. **and 7.** These two columns together control how library data is to be displayed in the **Hierarchy** and **OTU** panes.
   - **Show Libraries** shows all of the active libraries in a workspace.
   - **Show Sorted Libs** displays all libraries in a workspace after sorting and annotating library names by metadata selected through the **View → Sort Libraries** function.
   - **Show Lib Groups** shows the groups active and selected by the **Group → Group Library** function.
   - **All Libraries** shows all libraries in the dataset.
   - **Selected Libraries** shows just the subset of libraries selected by the **Current Filter**.
   - The value shown below **Selected Libraries** shows the number of libraries selected when **Selected Libraries** is chosen.
8. The four buttons in the eighth column (from top to bottom) allow the direct selection of figures created in any workspace in the project (**Figures**), a one click creation of a new workspace from the current workspace (**Clone Workspace**), a one click **Save** of all data within a project, and a one click shutdown of all workspaces in a project via **Close Project** (the user is first prompted to save any unsaved modifications to the project). All active windows closed by **Close Project** will be re-opened exactly as shown when the project is re-started.

Data display panes
1. The **Hierarchy Pane**: This pane allows the user to explore a data set in terms of hierarchical levels by which the user has organized the sequences in a dataset, such as Domain, Phylum, Class, Order, Family, Genus, and Species. These categories are classifier dependent and need not be phylogenetic/taxonomic classifications (e.g., for non-16S rRNA datasets). This pane shows a hierarchical summary of the data imported into the project, with levels selected as rows on the left side of the pane. Initially, data are presented as sequence counts for particular taxonomic levels, displayed for all libraries (the column "Total") or broken down library by library.
2. The **OTU Pane**: The left side of this pane shows an alphabetical list of all of the OTUs found in the data selected for the workspace. The "Total" column shows the representation of sequences in each OTU in all samples, and the rest of the pane presents data for individual libraries. This pane displays the data that will go into the analysis functions of Explicet or other statistical tools such as SAS or R via the **Export → OTU** function.

VI. Project Flow

The following outline represents a typical workflow for a project in Explicet. For more detailed information on this process, please view the Explicet Tutorial. The tutorial and associated data are obtainable from the website: www.explicet.org

```
┌─────────────────────────────────────────┐
│      Import Taxonomy Data for a Project  │
│              See page 23.                │
└─────────────────────────────────────────┘
                    │
                    ▼
┌─────────────────────────────────────────┐
│      Import Metadata for the Project     │
│              See page 30.                │
└─────────────────────────────────────────┘
                    │
                    ▼
┌──────────────────────────────────────────────────┐        ┌────────────────────────────────────┐
│         Adjust the Display as Necessary           │◄───────│   Repeat from this Step Until Done   │
│ Adjust display format (see page 33), sort data    │        └────────────────────────────────────┘
│              (see page 34).                       │
└──────────────────────────────────────────────────┘
                    │
                    ▼
┌──────────────────────────────────────────────────┐
│ Create a Subset of the Project Data for a Specific Analysis │
│         Create a workspace (see page 38).         │
└──────────────────────────────────────────────────┘
                    │
                    ▼
┌──────────────────────────────────────────────────┐        ┌────────────────────────────────────┐
│         Analyze Data and Save Figures             │        │            Edit Figures            │
│ Select data (see page 39), create figures (see    │◄───────│ To edit pre-existing figures, go   │
│ page 45), run statistical tests (see page 52).    │        │ directly to the desired figure and │
└──────────────────────────────────────────────────┘        │  modify as necessary (see page 51).│
                                                             └────────────────────────────────────┘
```

Figure 3. Typical flow for a project in Explicet.

Copyright and GPL License

Installation

I. Microsoft Windows

A. Install Package
Explicet installer files
Copyright and GPL license file
Explicet handbook
Explicet tutorial
Explicet tutorial data: OTU import file
Explicet tutorial data: metadata import file
Release notes
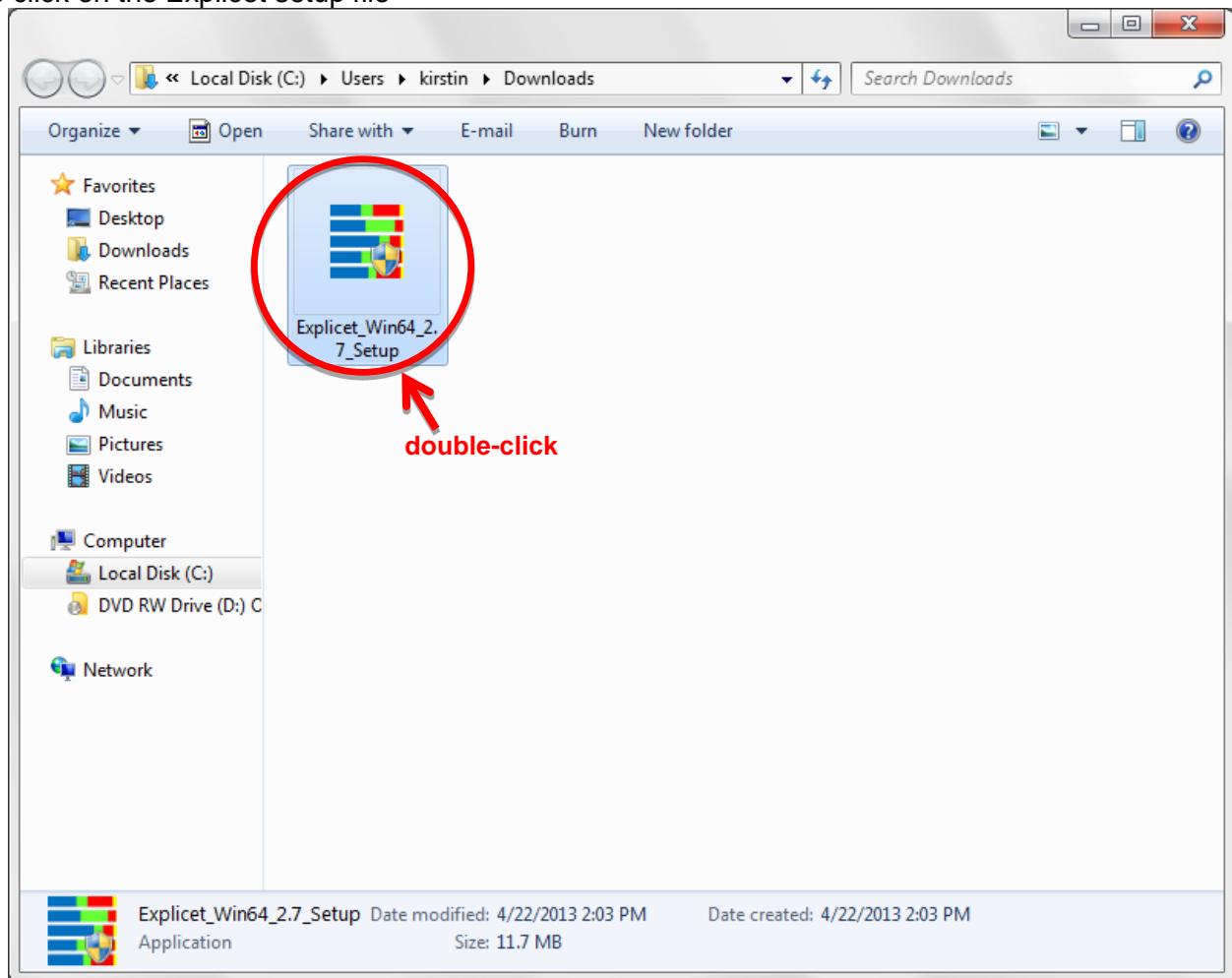
B. Supported Platforms
Windows Vista, Windows 7, Windows 8: Explicet_Win64
Windows XP: Explicet_Win32

C. Installation
Download the platform-appropriate installer: Explicet_Win64_##_Setup.exe (## is the version number)
Open the containing folder of the download
Double click on the Explicet setup file
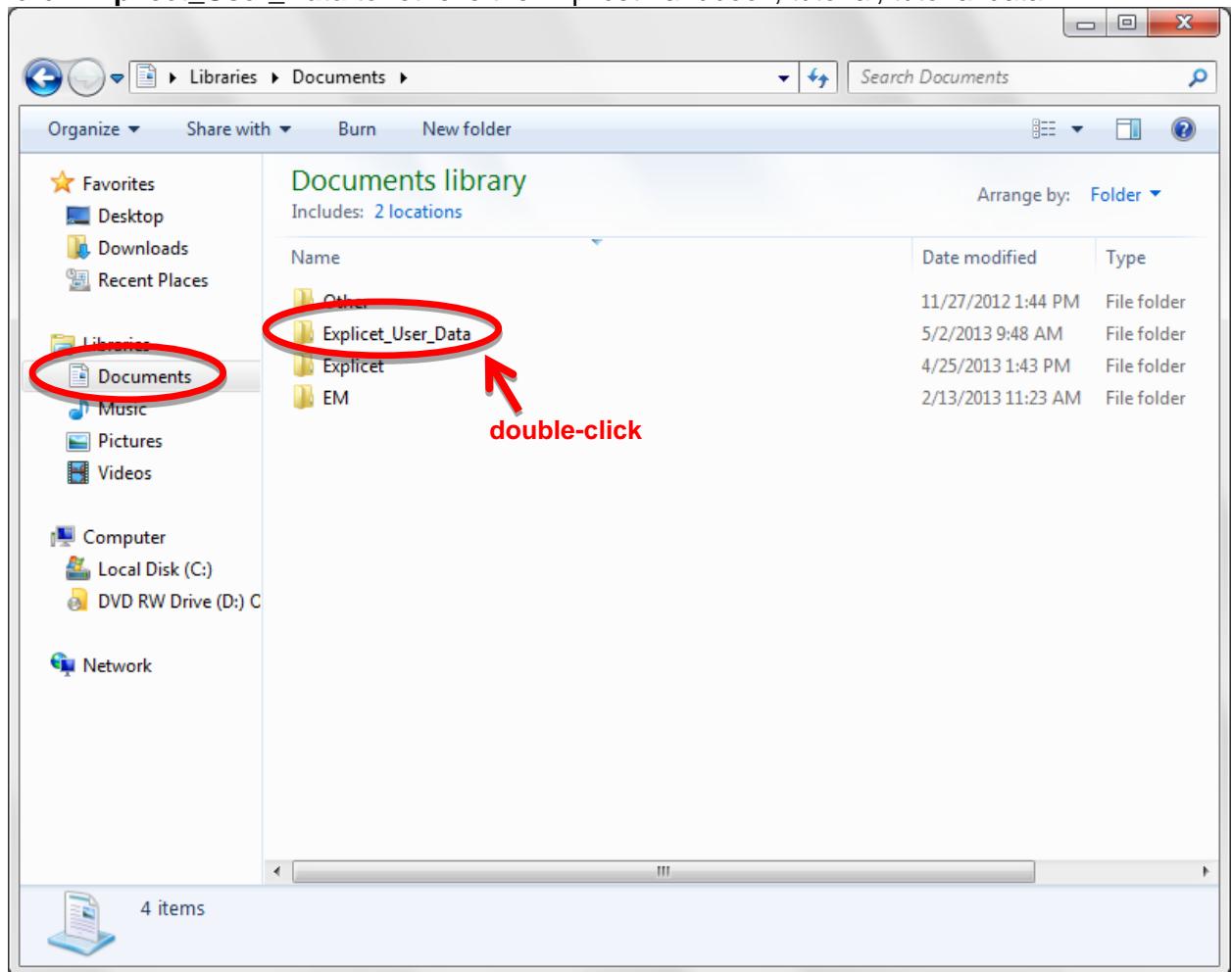
Follow the instructions on the Explicet **Setup Wizard**

D. Explicet Documentation
To access the Explicet documentation, click on **Documents**
Double-click **Explicet_User_Data** to retrieve the Explicet handbook, tutorial, tutorial data

II. Macintosh OS

A. Install Package

Explicet installer files
Copyright and GPL license file
Explicet handbook
Explicet tutorial
Explicet tutorial data: OTU import file
Explicet tutorial data: metadata import file
Release notes

B. Supported Platforms
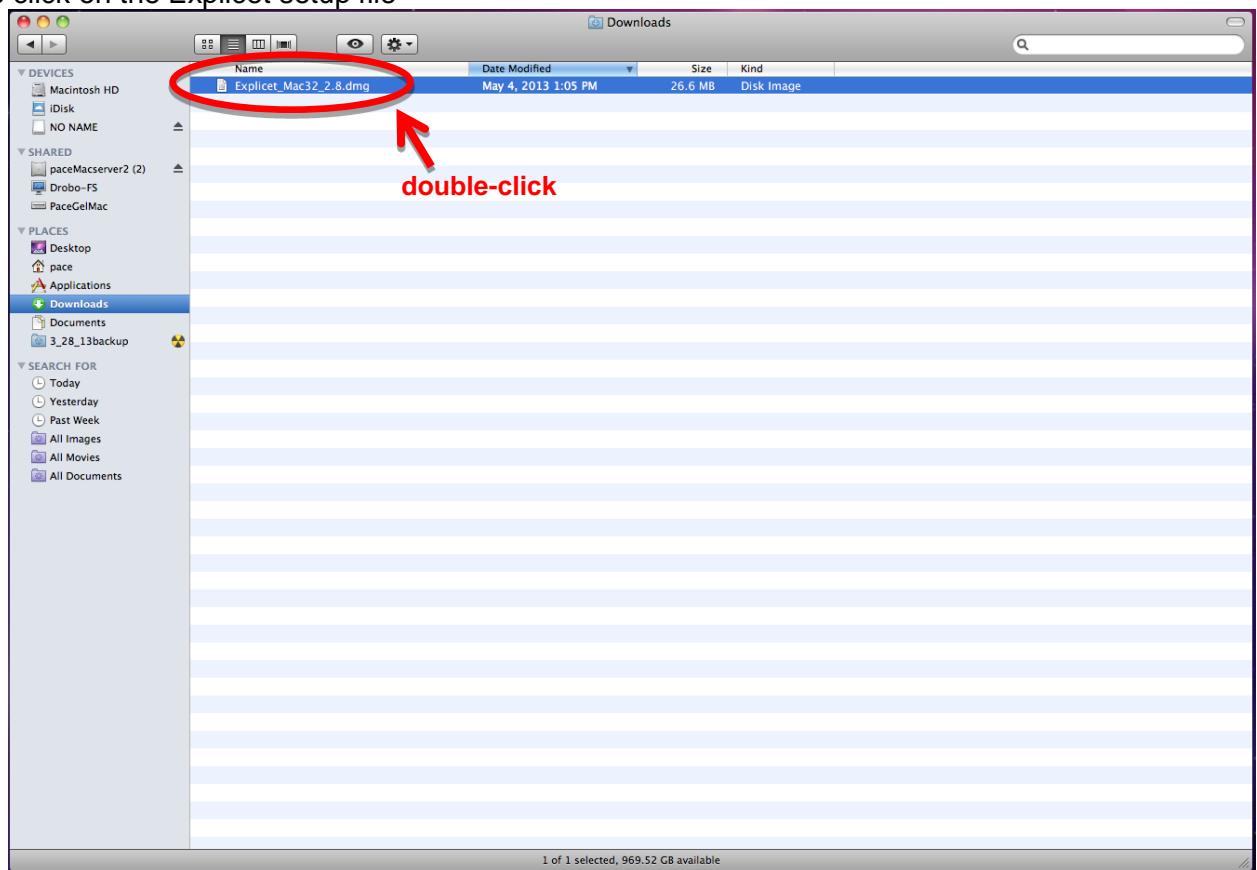
Lion (10.7), Mountain Lion (10.8): Explicet_Mac64
Snow Leopard (10.6): Explicet_Mac32

C. Installation

Download the platform-appropriate installer: Explicet_Mac32_##.dmg (## is the version number)
Open the containing folder of the download
Double click on the Explicet setup file
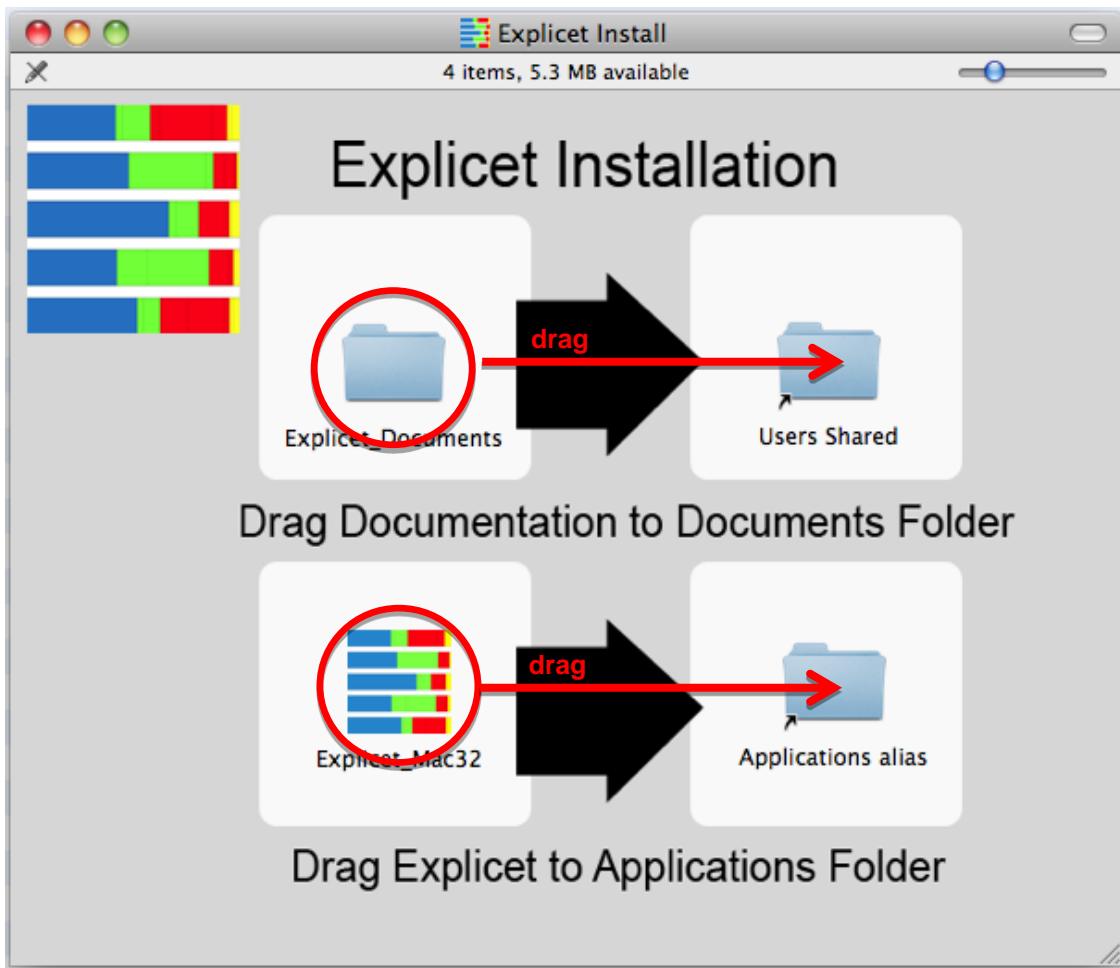
A graphic will appear
Drag the Explicet_Documents folder into the **Users Shared** folder
Drag the Explicet icon into the **Applications** folder
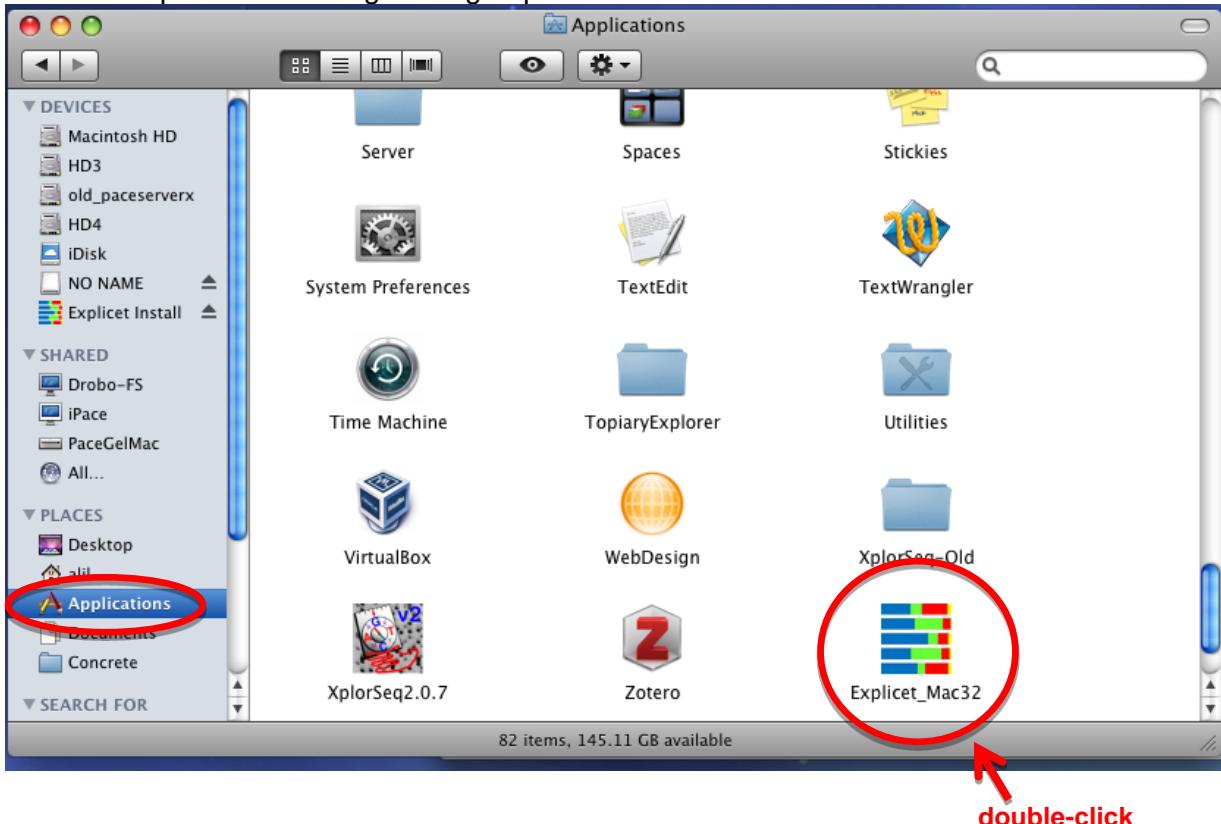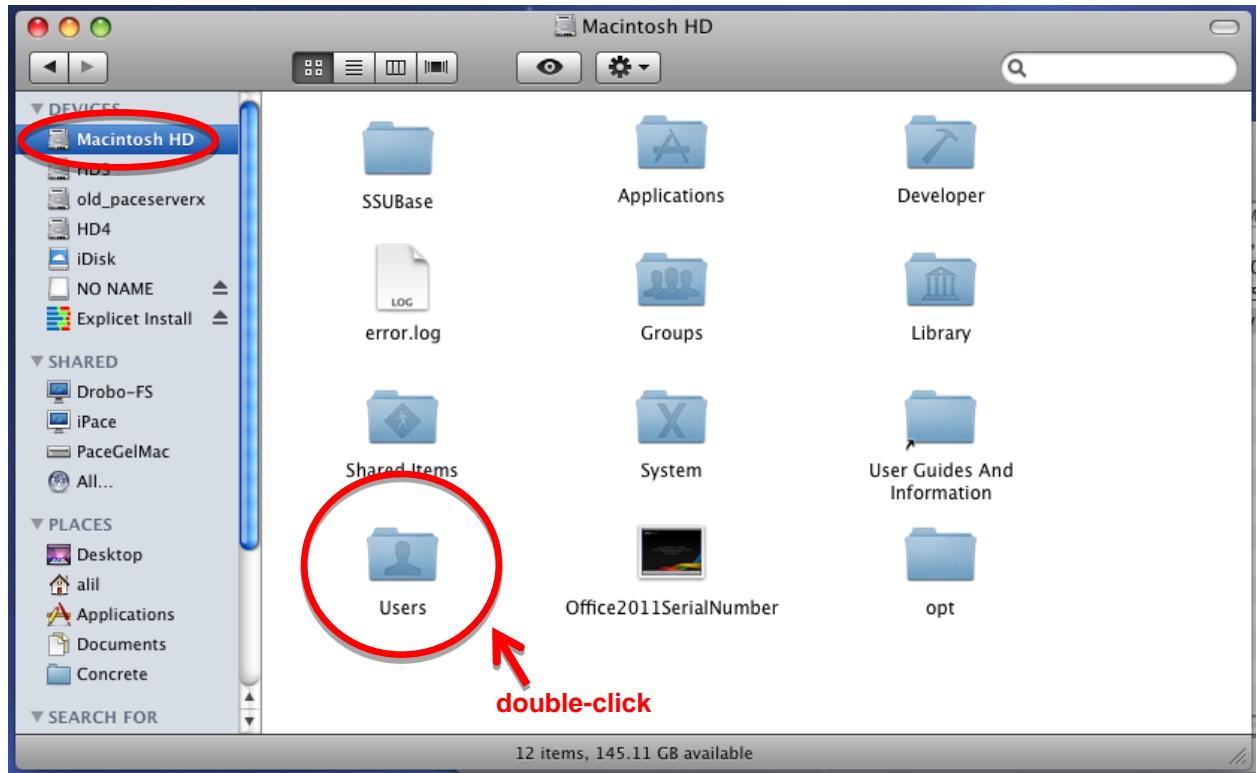
Open **Applications**
Double-click the Explicet icon to begin using Explicet
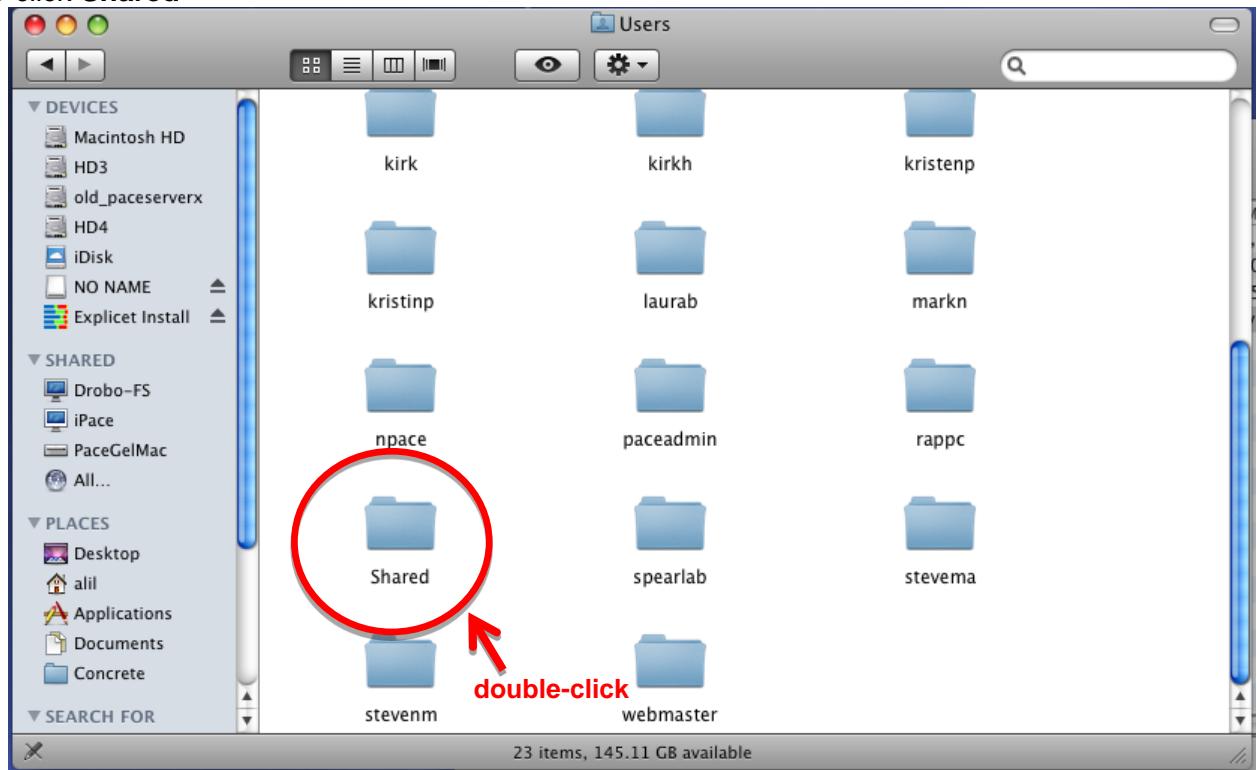
double-click

D. Explicet Documentation
To access the Explicet documentation, click on your hard drive
Double-click **Users**
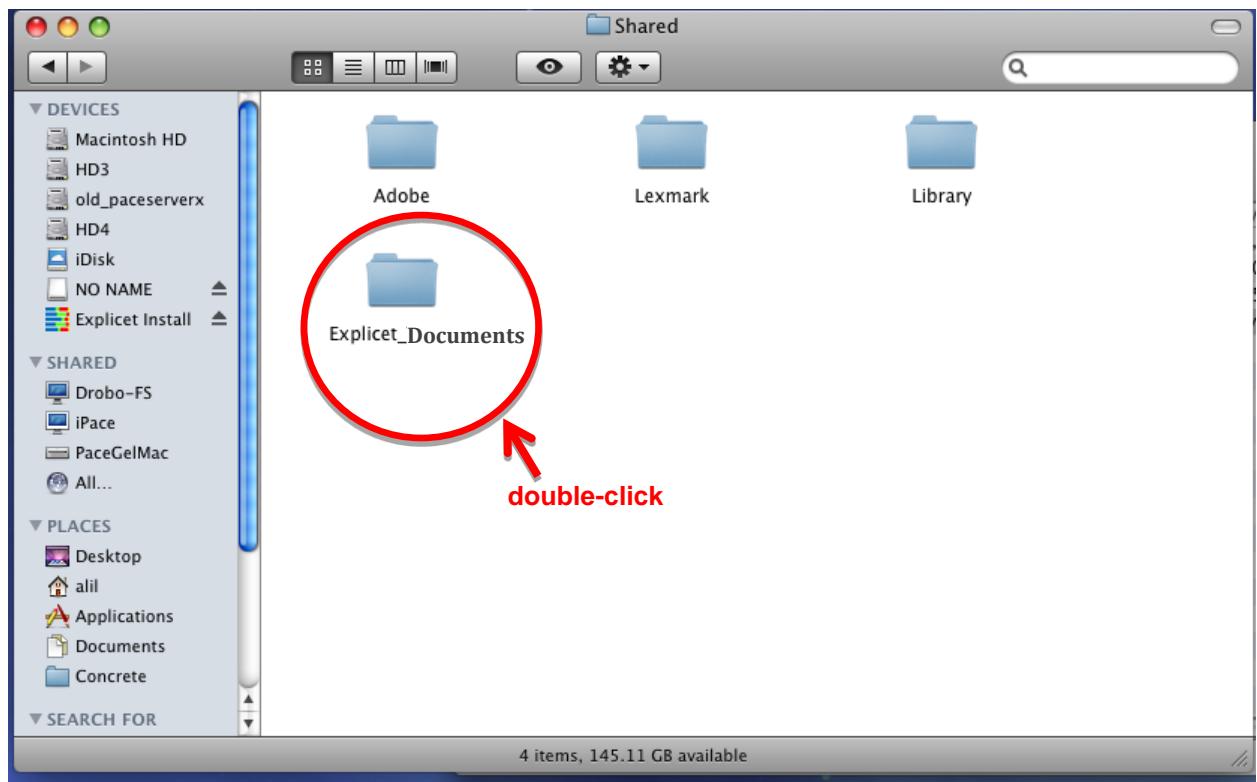
Double-click **Shared**

Double-click **Explicet_Documents** to retrieve the Explicet handbook, tutorial, tutorial data

double-click

III. Linux

Explicet for Linux is supplied as an executable which is statically linked with the required Qt and Qwt libraries. The executable has been tested extensively on CENTOS (RHEL) 5.x and 6.x.

An example of an install for Explicet on Linux follows.

First decompress the provided Explicet tar ball:

    tar –xvzf Explicet-Linux64_X.X.tar.gz

Which will provide the following:

    64 bit executable
    Copyright and GPL license file
    Explicet handbook
    Explicet tutorial
    Explicet tutorial data: OTU import file
    Explicet tutorial data: metadata import file
    Release notes

Create a symbolic link to the uncompressed Explicet binary in a folder known to be within the scope of the current path settings. For example, assuming the Explicet Linux executable is in /home/chuck, first make sure the execute bit of the application is set:

    chmod +x Explicet_Linux64_X.X

Now, as root (or via sudo), make a symbolic link:

    ln –s /home/chuck/Explicet_Linux64_X.X /usr/local/bin/explicetX.X

Alternatively, add the Explicet application to the path, or simply start Explicet in the local directory by typing:

    ./Explicet_Linux64_X.X

Condensed Quick-Start Example

The following guide presents a few quick examples of Explicet capabilities. For a more thoroughly documented example on the Explicet project process, please view the Explicet Tutorial. The tutorial and associated data are obtainable from the website: www.explicet.org

Open Explicet
A pop-up window will open with several different options
Click **Create Project**
Enter **Project Name**, "Tutorial_HSM", when prompted
Click **OK**

I.   Import the OTU table.
File → Import → File → OTU Table Counts
Select "Tutorial_HSM_OTU_2_Explicet"
Click **Open**
Another pop-up window will open
Click **Import**

II.   Import metadata for the OTU table.
File → Import → Metadata
Select "Tutorial_HSM_Metadata"
Click **Open**
Another pop-up window will open
Click **Import**
Another pop-up window will open
Click **Done**

III.   Save the project.
Click **Save** button in upper right corner of current workspace window
Enter desired file name (default file name is project name with an "_Explicet_Project" extension) and location
Click **Save**

IV.   Create a pie chart.
Tools → Plot → Pie Chart
A new window will appear with the hierarchical data available in the workspace
Holding "Shift", select all of the classes (Acidobacteria through Verrucomicrobia)
Click **Add to Pie**
Click **Plot**
A new window will appear containing pie chart display options
To display a single pie chart that includes all libraries' data, select the **30 Total Libraries** checkbox
Click **OK** to view the pie chart
Click **Done** to return to the pie chart setup window
Click **Save As Figure** in lower right corner of window
Enter desired figure name
Click **OK**
Click **Done** to return to the current workspace window
(The figure can always be recalled by clicking **Figures** button in upper right corner of workspace window)

V. Create a workspace (a subset of libraries and OTU lines).

File → New → Workspace
Enter "Back" workspace name when prompted
Click **OK** to create the new workspace

Data → Select Libraries
New pop-up window appears for creation of filters
Click **New** on far right side of window
Enter "Back" filter name when prompted
Click **OK**
"Back" appears in upper left corner of window
Click **Add** on right side of upper pane
Use the first pull-down menu to select "Anatomy" (metadata to filter by)
Use the second pull-down menu to select filter "contains" (operator)
Enter "back" into **Value**
To apply filter, click **Select** in upper right corner of window
Click **Save Filter** on far right side of window to keep the filter
Click **Done**
The workspace now only displays libraries sampled from the back.

VI. Compute beta-diversity (heatmap of Morisita-Horn matrix).

Tools → Analyze → Beta Diversity → Morisita-Horn
A new window appears containing a comparison matrix with the calculated Morisita-Horn similarity values
Select **Selected Libraries** in the upper left corner of the window
Click **Plot** on lower right side of window
A heatmap appears displaying a comparison of the OTUs between the "back" library samples
Click **Done** to return to the heatmap setup window
Click **Save As Figure** in lower right corner of window
Enter desired figure name
Click **OK**
Click **Done** to return to the current workspace window
(The figure can always be recalled by clicking **Figures** button in upper right corner of workspace window)

VII. Create an x-y plot of Species observed, Sobs.

Tools → Analyze → Alpha Diversity
A new pop-up window will appear
To create curves, deselect **Single statistic at Rarefaction point only**
To create smooth curves, change **Bootstrap Size** to 1000
Click **Bootstrap**
Click **Plot** on lower right side of window
A pop-up will appear with all the diversity measures and library names
Select **Sobs** (the number of OTUs observed in a library)
Click **OK**
A plot of the rarefaction curves for the selected measures and libraries will appear
Click **Done** to return to the alpha diversity setup window
Click **Save As Figure** in lower right corner of window
Enter desired figure name
Click **OK**
Click **Done** to return to the current workspace window
(The figure can always be recalled by clicking **Figures** button in upper right corner of workspace window)

Comprehensive User Guide

I.   Import OTU Data

The Explicet program provides a GUI for exploring OTU data, but does not generate the OTU data. Rather, taxonomic data for a project can be imported from a variety of sequence processing software programs including the RDP Classifier [2], ARB NDS [5,7], Mothur [10], and Qiime [1], and in multiple formats. Data import is fully incremental, so data may be imported at any time.

Note: The import format, date, time, and file location of OTU data are logged in **Edit → Project Settings**.

A. OTU Data File Format
The import file must be a delimited file (i.e., delimited by tabs, commas, or spaces).

B. Import a File
An import file contains multiple libraries (a.k.a. samples) in a single file.

File → Import → File →…

1. Three-line (legacy)
One file with three lines for each OTU result:
   (line 1) library name and sequence name separated by a tab
   (line 2) *this second line is ignored*
   (line 3) OTU names separated by slash/tab/semi-colon/colon/comma/period/space

   Example:
   TPN0001B <tab> TPN0001B_000001
   A
   /Root/Bacteria/"Bacteroidetes"/"Bacteroidia"/"Bacteroidales"

2. Two-line (fasta style)
Requires two files...

File 1 = file with two lines for each OTU result:
   (line 1) >sequence name
   (line 2) OTU names separated by slash/tab/semi-colon/colon/comma/period/space

   Example:
   >TPN0001B_000001
   /Root/Bacteria/"Bacteroidetes"/"Bacteroidia"/"Bacteroidales"

File 2 = file mapping sequences to libraries, with one line for each OTU result:
   (line 1) sequence name and library name separated by a tab

   Example:
   TPN0001B_000001 <tab> TPN0001B

   Note: It is the user's responsibility to create the mapping file, for instance using a spreadsheet.

3. One-line (ARB NDS)
Requires two files...

File 1 = file with one line for each OTU result - such as that emitted by the NDS export function of ARB:
   (line 1) sequence name and OTU name separated by a tab

   Example:
   TPN0001B_000001 <tab> /Root/Bacteria/"Bacteroidetes"/"Bacteroidia"/"Bacteroidales"

File 2 = file mapping sequences to libraries, with one line for each OTU result:
   (line 1) sequence name and library name separated by a tab

   Example:
   TPN0001B_000001 <tab> TPN0001B

   Note: It is the user's responsibility to create the mapping file, for instance using a spreadsheet.

4. RDP Classifier
Explicet handles both local and web formats. Requires two files...

File 1 = file with one line for each OTU result:
   (line 1) sequence name and OTU name with bootstrap scores

   This file is formatted as exported by RDP. Please reference the website for more information:
   http://rdp.cme.msu.edu/classifier/classifier.jsp

   Upon import, the user has the option to change the **Bootstrap Threshold** for the OTUs being imported. The import default on Explicet is 80%. For more information on recommended bootstrap scores, visit the RDP website listed above.

File 2 = file mapping sequences to libraries, with one line for each OTU result:
   (line 1) sequence name and library name separated by a tab

   Example:
   TPN0001B_000001 <tab> TPN0001B

   Note: It is the user's responsibility to create the mapping file, for instance using a spreadsheet.

5. OTU Table Counts
This is the most compact and efficient way to import OTU files. Instead of listing each individual sequence, this is one file which contains each OTU name listed once and a count for each time it appears in each library:
   (top line) library names as column headings separated by a tab
   (following lines) OTU name and library counts separated by a tab

   Example:
   OTU Name <tab> Total <tab> HV1-1-BtRSc <tab> HV10-BtRSc <tab> HV2-1-BtRSc
   root <tab> 9,049 <tab> 326 <tab> 198
   Bacteria <tab> 2 <tab> 2 <tab> 0
   Bacteria/Acidobacteria/Acidobacteria/RB41 <tab> 1 <tab> 0 <tab> 0

   Note: OTU table count files are exported from Explicet, Mothur, and Qiime.

C. Import a Directory
A directory contains a separate file for each library. The names of each file (less the file extension) will be used as the library name for the sequences within the file.

File → Import → Directory →…

1. Two-line (fasta style) / One-line (ARB NDS) / RDP Classifier

Each individual file is formatted as described above in "Import a File"; however, each file name (less the file extension) is the library name (instead of a mapping file).

II. Moving Data to and from Other Toolsets: Qiime, R

A. Moving Data from Qiime to Explicet

Explicet can import data from Qiime via Qiime's "classic" OTU table format. More recent versions of Qiime require conversion from their internal .biom format to the classic OTU table format as follows:

```
convert_biom.py -i table.biom -o table.from_biom_w_taxonomy.txt -b --header_key taxonomy
```

Additional information on the conversion process may be found here:
http://biom-format.org/documentation/biom_conversion.html

Many thanks to Dr. Lee Stanish for assistance in testing this .biom conversion process.

B. Moving Data from Explicet to R

Explicet data can be moved to the R package for more advanced statistical analysis. The Explicet tutorial example will be used to illustrate the steps involved in moving data from Explicet to R. (Note: R packages are written by a diverse collection of individuals, and particular functions may require additional data manipulation that is not shown below.)

1. Data Export from Explicet

Assuming you have built an Explicet project from the Explicet Tutorial, export the following:

An OTU file of *count* data, selecting the **Export to R** checkbox (**File → Export → OTU**). Name this file hsm_otu_cts.txt. Sort the OTUs from greatest to least abundance before exporting so that it is easier to pick out the most abundant OTUs in R. For full information on exporting OTU tables, see page 44, "OTU Table".

A metadata file, exported as usual (**File → Export → Metadata**) and named hsm_meta.txt. For full information on exporting metadata, see page 31, "Export Metadata".

2. Data Import into R

Open R and change the working directory to the directory storing hsm_otu_cts.txt and hsm_meta.txt.

Import the OTU file by entering:
```
hsm_otu_cts <- read.table("hsm_otu_cts.txt",header=TRUE, na.strings = "na", sep="\t")
```

Import the metadata file by entering:
```
hsm_meta <- read.table("hsm_meta.txt",header=TRUE, na.strings = "na", sep="\t")
```

3. Pre-process OTU File

Create an object storing the OTU names:
```
hsm_otu_nms <- hsm_otu_cts[, 1]
```

Remove OTU names from OTU table:
```
hsm_otu_cts <- hsm_otu_cts[, -1]
```

Transform data table so that each row is a different subject:
```
hsm_otu_cts <- t(hsm_otu_cts)
```

Convert to a data matrix:
```
hsm_otu_cts <- as.matrix(hsm_otu_cts)
```

Note: The previous three commands can be simplified into the following one command:
```
hsm_otu_cts <- as.matrix(t(hsm_otu_cts[, -1]))
```

As a check, enter the commands "ncol(hsm_otu_cts)" and "nrow(hsm_otu_cts)" to output the number of columns (131) and rows (30) in the resulting data structure.

4. Pre-process Metadata File
Enter the commands "ncol(hsm_meta)" and "nrow(hsm_meta)" to output the number of columns (6) and rows (30). The number of rows in the metadata file should match the number of rows in the OTU files, since rows specify the libraries in the datasets.

5. Quick Analysis Using Vegan
Assuming that "vegan" has been installed in your R environment…

Load the vegan package: library("vegan")

To determine whether the skin microbiome differs by Anatomy, enter:
        adonis(hsm_otu_cts ~ hsm_meta$Anatomy, method="bray", permutations = 1000)

The output, shown below, indicates a significant association between Anatomy and skin microbiome. Note: The p-value, under the heading Pr(>F) is calculated using a label-permutation test, so it will differ from run to run.

Adonis output:
Call:
adonis (formula = hsm_otu_cts ~ hsm_meta$Anatomy, permutations = 1000, method = "bray")

Terms added sequentially (first to last)

	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)
hsm_meta$Anatomy	3	0.85764	0.285879	4.4542	0.33947	0.000999 ***
Residuals	26	1.66874	0.064182		0.66053	
Total	29	2.52637			1.00000	

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

III. Edit Libraries and OTU Names

A library is a set of OTUs that represent the results of classifying sequences derived from a discrete sample/specimen.

Caution: Performing any of the following actions will permanently alter primary data in the project. Users *cannot* undo Rename Libraries, Remove Libraries or Remove OTU Names.

A. Rename Libraries

Renaming libraries is a *permanent* change to any project!

1. Delimited Text File

To rename many libraries at once, create a file that remaps the current names of libraries. The file should contain one line for each library name. The file containing the new library names must be a delimited text file (old library name and new library name separated by a tab, space, or comma). Library names cannot have spaces in them.

Example:
Sp02z56_00034 <tab> Sp02z56 (re-maps library Sp02z56_00034 to library Sp02z56)

Edit → Project Settings
Click **Rename Libraries**
Select rename library file
Click **Open**

2. Edit in Metadata

Instead of importing new library names from an outside source, the user can edit library names from within Explicet.

Data → Metadata
Click on the library name in the **Assigned Metadata** pane
Type over the existing library name
Click **Apply** to keep the changes to the library name
Click **Done** to return to the project

Note: The rename date, time, old name, and new name of a renamed library are logged in **Edit → Project Settings**.

B. Remove Libraries

Removing libraries is a *permanent* change to any project!

Edit → Project Settings
Click **Remove Libraries**
A pop-up window will open
Click on library name in left panel to select
· Several libraries can be selected for removal by clicking the first library, then Shift-click on the last library. Non-sequential libraries may be selected (or deselected) via Ctrl-click.
Click **Select** button between panels
Repeat until all undesired libraries are moved to the right panel
Click **Remove** in upper right corner of window
A pop-up message will appear to warn you that you are about to delete data
Click **OK** to remove libraries

Note: The deletion date, time, and name of a removed library are logged in **Edit → Project Settings**.

C. Remove OTU Names

Removing OTU names is a *permanent* change to any project!

Edit → Project Settings
Click **Remove OTU Names**
A pop-up window will open
- If an OTU name is highlighted in the workspace window, it will appear in the OTU name edit box.

Under **Choose OTU names that**, use the pull-down menu to select OTU name filter operator
Enter the OTU name that you wish to remove into the field under the pull-down menu
Click **Select**
OTU names which meet the conditions entered will appear in the bottom pane
Select the OTU names that you wish to remove
- Several OTU names can be selected for removal by clicking the first OTU name, then Shift-click on the last OTU name. Non-sequential OTUs may be selected (or deselected) via Ctrl-click.

Click **Remove** in upper right corner of window
A pop-up message will appear to warn you that you are about to delete data
Click **OK** to remove OTU names

Note: The deletion date, time, and name of removed OTU names are logged in **Edit → Project Settings**.

IV. Metadata

Metadata is information about the source of the DNA sequences (e.g., GPS coordinates, pH, patient, disease status, age, weight).

A. Metadata File Format

The top row must be labels that are the names of the metadata categories for the project (e.g., library, specimen, time). One column must be library names exactly as they appear in the OTU file. This column maps the library names to the metadata values for that library. The column position of the library names within the metadata file does not matter, but the column must be present. The column fields must be delimited by either tabs, commas, or spaces.

B. Import Metadata

Metadata import is fully incremental, so new metadata may be loaded at any time during the life of the project.

Note: The import date, time, and file location of metadata are logged in **Edit → Project Settings**.

File → Import → Metadata
Select file
Click **Open**
A pop-up window will open
The upper left pane of the window shows the metadata fields found by Explicet in the metadata file
- When **Libraries not found** is selected, any libraries found in the metadata import file that are not in the project appear in the upper right pane.
- When **Libraries found** is selected, any libraries found in the metadata import file that already exist in the project appear in the upper right pane.
- By default, **Add missing libraries to the project** is deselected so that metadata import doesn't force empty libraries into projects. By selecting **Add missing libraries to the project**, any library found in the metadata import file that is not in the project will be added to the project and populated with imported metadata. Since there are no sequences associated with these libraries, the data columns will be filled with zeros in the hierarchy and OTU panes.
- Metadata in the selected file that does not match the format of metadata already in the project appears in the bottom pane. For example, if "Age" is a metadata category containing numerical data, attempting to import text data into the "Age" category will be noted in the bottom pane.
In upper left pane, select the column which contains the library name
Click **Import**

A new pop-up window will open which displays the imported metadata. This window can be re-accessed via **Data → Metadata**.

C. Add New Metadata

Instead of importing metadata from an outside source, the user can create new metadata from within Explicet.

Data → Metadata
A window with three sections will open
- The left pane: **Defined Metadata**, the right pane: **Assigned Metadata**, the bottom area: **Metadata Definition**. The bottom section, **Metadata Definition**, allows creation of new metadata fields.
Enter the desired name of the new metadata category next to the box labeled **Name**
Choose the **Type** of metadata from the drop down menu
- **String that may be more than 8 characters**: words or strings of text longer than 8 characters (e.g., umbilicus)
- **String that is 8 characters or less**: words or strings of text 8 characters or less (e.g., male)

- **64 bit Integer**: whole number/number with no fractional part (e.g., 21, 4, or -2)
- **Double precision number**: number with a fractional part (e.g., 3.75 or 5.5)

(optional) For integers or numbers, an upper and lower bound can be defined…

(optional) If there are only a few, distinct metadata values for the new definition, enter them as enumerated values; type each value under **Enumerated Values**, and click **Add Value**

- To remove an enumerated value, select the value and click **Remove Value**.
- To clear all enumerated values for a particular definition in one click, click **Clear Values**.

Click **Add** in bottom right corner to add the new metadata definition to **Defined Metadata** list

Select new metadata definition in the **Defined Metadata** pane

Click **Add** button between panes to add it to **Assigned Metadata**

For each library in the **Assigned Metadata** pane, enter the corresponding value

- If **Enumerated Values** were entered, the values will appear in a drop down menu for each library.
- To fill values for multiple libraries at once, use the **Copy** and **Paste** functions.
- To select whole rows, click on the first value to select, then Shift-click on the last value; to select whole columns, click on the title of the column. Click the **Copy** button (the values are copied to an internal clipboard). To paste the copied values to other cells, select the cells you want to paste to, and click the **Paste** button. Note: If the clipboard contains a multi-row and/or multi-column selection, the number of rows and columns to paste to must match the number of rows and columns copied to the clipboard. If they don't match, an error message will appear, and nothing will be copied.
- To copy one value to multiple cells, select the cell to copy, click **Copy**. Select the cells to paste to, click **Paste**.

Click **Apply** to keep the changes; click **Done** without clicking **Apply** to discard the changes

Note: All operations are performed on the selected libraries displayed in the **Assigned Metadata** pane. You can show **All Libraries** in this pane or click the **Selected Libraries** radio button to operate on the libraries chosen by the current workspace filter.

D. Modify Current Metadata

To set or change values in the **Assigned Metadata** pane, click on the value to change, and type in a new value. If the metadata values are enumerated values, a drop down from which to pick one of the **Enumerated Values** will appear.

To modify an existing metadata definition, click on it in the **Defined Metadata** pane. Its definition will show in the box labeled **Metadata Definition**. Make any desired changes; change the **Name**, the **Type**, add **Upper**/**Lower Bounds**, or add **Enumerated Values**, then click **Replace**. Keep the changes by clicking **Apply**.

To create new metadata from existing metadata, select the metadata in the **Defined Metadata** pane. The name MUST be changed. Then make any other changes to **Type**, **Bounds**, or **Enumerations**, and click **Add**.

Note: All operations are performed on the selected libraries displayed in the **Assigned Metadata** pane. You can show **All Libraries** in this pane or click the **Selected Libraries** radio button to operate on the libraries chosen by the current workspace filter.

E. Export Metadata

Metadata is exported as a tab-delimited (.txt) file. There are 2 different ways to export metadata:

1. **File → Export → Metadata**
   Enter desired metadata output file name and location
   Click **Save**
2. **Data → Metadata**
   Click **Export** in upper right corner of pop-up window
   Enter desired metadata output file name and location
   Click **Save**

F. View Metadata
Data → Metadata

A window with three sections will open. The left pane, **Defined Metadata**, has the libraries and columns describing each metadata point associated. The right pane, **Assigned Metadata**, has the metadata titles. The bottom area, **Metadata Definition**, allows you to create new metadata fields (instead of importing them from elsewhere).

G. Remove Metadata Assignment
This will remove the metadata assignment to the selected libraries shown in the **Assigned Metadata** pane.

Data → Metadata
A pop-up window will appear
Select unwanted metadata column in the right, **Assigned Metadata**, pane of the window
Click **Remove** button between panels
A pop-up message will appear to warn you that you are about to delete data
Click **OK** to remove metadata

Note: Explicet does not store the metadata values for each library once it is removed from the **Assigned Metadata** pane. Therefore, if you decide to **Add** the metadata item back to **Assigned Metadata** later, the values for each library will appear blank.

H. Remove Metadata from Project
This will remove a metadata definition from the project.

Data → Metadata
A pop-up window will appear
Select unwanted metadata item in the **Defined Metadata** pane of the window
Click **Delete** button to the right of the **Metadata Definition**
A pop-up message will appear to warn you that you are about to delete data
Click **OK** to remove metadata

V. Display

A. Hierarchy, OTU, or Both

1. Hierarchy

In **Hierarchy** mode, the data collapses into OTUs with triangular expansion buttons. Clicking on an expansion button expands the selected taxonomic level to the next lower level. This generates an easy-to-read display that can be expanded and compressed to investigate comparisons between libraries or metadata groups. The number of items in the lineage lines is determined by the input file, i.e. the depth of classification during pipe run on raw sequence data. Clicking on the OTU category will highlight the line across the entire dataset. **Hierarchy Level** (see page 34) sets the default of how many levels of hierarchy are displayed.

2. OTU

In **OTU** mode, each taxonomic classification is listed individually, often resulting in very long OTU names that use large amounts of screen space. To make the data more tractable in this setting, use the **OTU Start**, **OTU Width**, and **OTU Last** functions (see page 33 for details). The number of categories in the lineage lines is determined by the input file, i.e., the depth of classification provided by the classification analysis tool run on the raw sequence data. Clicking on the OTU name will highlight the line across the entire dataset.

3. Both

Selecting **Both** creates two panes on the workspace screen. The upper pane shows the Hierarchy, and the lower pane shows the OTUs.

B. Raw and Normalized Data Options

1. Counts

This option shows the raw sequence data counts (integers).

2. % of Library

Data is normalized by column (library or metadata grouping). This is generally the most useful setting for comparing sequence distributions between samples or groups of samples.

3. % of Total

Data is normalized to the entire OTU count for the total dataset.

C. OTU displays

1. OTU Start

This allows the user to control the taxonomic level at which to start the lineage lines in the OTU pane display. For example, rather than every sequence starting at the domain level 1 for domain (Bacteria/Eukarya/Archaea), the user can select **OTU Start** at level 2 for phylum (Acidobacteria/Proteobacteria/etc.), or at level 3 for subphylum (Alphaproteobacteria/Betaproteobacteria/etc.).

2. OTU Width

This option limits the number of taxonomic categories displayed in the OTU name. The number of taxonomic categories can be selected by entering the desired taxonomic levels to display. For example, setting **OTU Start** at level 1, and **OTU Width** at 3, would produce the following: "Bacteria/Proteobacteria/Alphaproteobacteria…"

3. OTU Show Last

This option appends the last item in the taxonomic line onto an OTU name, independent of the values of **OTU Start** and **OTU Width**. The depth of taxonomic classification is determined by the input file. In the example above, **OTU Show Last** could be the genus level: "Bacteria/Proteobacteria/Alphaproteobacteria…/Paracoccus"

All OTU names can be forced to display ONLY the terminal category by checking this option and setting **OTU Width** to zero. One item OTUs (such as OTU Group names or OTUs that can only be traced to kingdom level, like "Bacteria") are considered the last OTU position; single name OTUs will disappear when **OTU Show Last** is deselected.

D. Hierarchy Level
Sets the default number of taxonomic levels expanded in the hierarchy pane.

E. Libraries

1. Show Libraries
This option will display the libraries by name and sort them alphabetically by name (e.g., AL123, EM321, RA964) from left to right.

2. Show Sorted Libraries
Sorted libraries have one or more metadata tag(s) pre-appended before the library name, separated by a colon. The libraries are then displayed alphabetically first by metadata tag(s), then by library name. This option allows the user to sort the library columns by metadata while still seeing all individual names as well (e.g., City1:AL124, City1:RA964, City2:EM321).   Often libraries and sequences are named with obscure codes, this mechanism allows the introduction of human readable text which may ease the interpretation of the project data. This human readable metadata is carried forward into all of the analyses and graphical displays within Explicet.

a) Sort Libraries
View → Sort Libraries
A pop-up window will open
In left panel, select the metadata descriptor you wish to sort by
Click **Add** button between panels
Name of metadata descriptor will appear in the right panel
- To change the sort order of an individual metadata, click on the current **Sort Order** value (**ascending/descending**), and select the desired sort order from the dropdown menu.
- To sort by more than one metadata item, pick multiple metadata descriptors from the list. Metadata items are sorted in the order they appear in the list.

Click **Sort** at top right of window to accept library sorting

b) Remove Library Sorting
If you wish to remove a library sorting, follow the same procedure, except select the undesired metadata field in the right panel and click **Remove** button between panels.

3. Show Library Groups
This option displays the results of creating groups of libraries with the **Group Libraries** command. Groups are formed by selecting libraries based on a common metadata field. The relative abundances of each selected library are averaged to form the values for the group. The **Counts** values displayed for groups is computed by using the averaged relative abundances for each taxonomic categories. For more information on how the **Counts** in library groups are calculated, and to create library groups, see page 39, "Group Data".

4. All Libraries
This option shows all libraries in the workspace, and the display is arranged according to the **Show Libraries** / **Show Sorted Libs** / **Show Lib Groups** selection.

5. Selected Libraries
It is often desired to look at a subset of the libraries within a project. In Explicet, this is done by first creating a filter, then using the **Selected Libraries** mode. In this mode, the user can examine data only from the libraries of interest (based on the criteria set in the filter), instead of from the entire dataset. Whereas **Sort Libraries** simply rearranges the view layout, **Select Libraries** excludes data from the view.   Select Libraries is often used in conjunction with the creation of a separate

workspace for the libraries of interest. For a description of how to select libraries, see page 39, "Select Libraries".

F. Sort OTUs

Sorting OTUs will arrange the data columns in the OTU pane by ascending or descending OTU abundance.

1. Sort OTU

Click on the library name in the OTU pane to sort by descending order based on the contents of that column (or **Total**). A small arrow will appear above the column title which indicates sort order.

Click the library name again to flip the sort order. The small arrow above the column title will switch to pointing upward, indicating that the OTU pane has been sorted in ascending order based on the contents of that column (or **Total**).

2. Remove OTU Sorting

There are 3 different ways to restore alphabetic sorting of the OTU pane:
1. Double click on the library name or **Total**
2. Hold Ctrl-click on the library name or **Total**
3. **View → Unsort OTU**

VI. Projects

Projects differentiate between various datasets (e.g., different studies) or between different versions of the data (e.g., different methods of assigning OTU names). Projects provide an organizing mechanism for user's data: both the OTU data and associated metadata. Each project is a single data file. The name of the current project is displayed in the upper left corner of each workspace window. Users may choose to use the data management capabilities of Explicet, which are shown in Figure 2 (page 9). As shown in the figure, projects may contain one or more workspaces. Workspaces are generally made up of subsets of the data contained in the full project and are wholly contained within the project. Projects and workspaces contain figures, which are tabular and/or graphical representations of data (e.g., a graph of species observed, Sobs, for the data in a project or workspace). All figures created within a project and its workspaces are globally available from the project or any workspace created within that project.

A. New Project
File → New → Project
Enter desired project name when prompted
Click **OK** to create the new project

B. Copy of Project
Clones all project-associated data, including OTU tables, filters, and figures.

File → New → Project from Current Project
Enter desired project name when prompted
Click **OK** to create a copy of the current project

C. Open Existing Project
File → Open → Project
Select file
Click **Open** to open project

D. Rename Project
Edit → Project Settings
Type over existing name in the top line of the pop-up window
Click **Apply** to rename current project

E. Save Project
There are 2 different ways to save a project:
1. Click the **Save** button in upper right corner of any workspace window.
2. **File → Save / File → Save As**

Note: Quick keys (e.g., Ctrl-s) are not currently supported in Explicet. Explicet does not auto-save, so remember to save often!

F. Close Project
There are 3 different ways to close out of Explicet:
1. Click the **Close Project** button in upper right corner of any workspace window.
2. **File → Close → Project**
3. **File → Exit**

Note: If you close a project with multiple workspaces open, the project is saved as-is. The next time you open the project, all workspaces will open exactly as in the previous session.

G. Project Settings
Project Settings provides a nice layout summarizing all of the project components.

Edit → Project Settings

- Edit the **Project Name**.
- If the current project is one of several different versions or one of several different studies on the same data, you can record the **Project Version** number.
- Record **Comments** about the project's specific aims, trends, notes, etc.
- When **Do not import Libraries whose total count is zero** is selected, any libraries with no members (e.g., the total number of OTUs in the library is 0) will not be added to the project.
- Projects may have libraries with no members if **Do not import Libraries whose total count is zero** is not selected. However, the libraries can be eliminated from computations if you pick the **Filter out Libraries whose total count is zero** option. In other words, if a library has no members, and **Filter out Libraries whose total count is zero** is selected, the library will not appear in the workspace window, Alpha Diversity, Beta Diversity, etc.
- By default, Explicet appends an "_Explicet_Project" extension to filenames in order to help distinguish a project file from other files. This default can be removed by deselecting **Append "_Explicet_Project" to Filename**.
- Edit the **Column delimiter for Export** (character(s) placed between columns); delimiter may be up to 6 characters, enter "tab" for tab.
- Adjust the **# of Decimal Places for Percents** in OTU displays.
- View the date and time the project was created.
- View the date and time the project was last saved.
- View the quantity and names of the project's associated **Workspaces**, **Filters**, **Figures**, **Metadata** tags, and **Libraries**.
- The bottom pane (labeled **Project Change Log**) tracks all major edits to the project data, including: OTU data imports, metadata imports, deletion of libraries, deletion of OTU lines, renaming of libraries.

Click **Save** to keep the changes
- Click **Restore** to revert changes made since the last **Save**. Caution: The **Restore** function does not apply to **Rename Libraries**, **Remove Libraries** or **Remove OTU Names**. Users *cannot* undo these actions! For more information on these functions, see page 26, "Edit Libraries and OTU Names".

VII. Workspaces

Workspaces allow the user to analyze subsets of the data that make up a project.   For example, if a project contains data from patients of different ages, several workspaces could be set up to handle specific age ranges. Similar to figures, workspaces are saved within the larger project, so they stay linked to the data from which they were created and do not create additional computer files.

Selected libraries (selected via filters) are saved to the larger project, but separate filters can be applied to different workspaces. For a description of how to select libraries, see page 39, "Select Libraries".

Library data may be combined into groups, which are local to the workspace in which they were created. To group libraries, see page 40, "Group Libraries".

Note: Libraries deleted from any workspace are also deleted from the larger project!

A. New Workspace
File → New → Workspace
Enter desired workspace name when prompted
Click **OK** to create the new workspace

B. Copy of Workspace
There are 2 different ways to create a copy of a workspace:
1. **File → New → Workspace from Current Workspace**
   Enter desired workspace name when prompted
   Click **OK** to create a copy of the current workspace
2. Click **Clone Workspace** button in upper right corner of any workspace window.

C. Open Existing Workspace
File → Open → Workspace
Select workspace, click **OK** to open workspace

D. Change Workspace
Click the radio button next to the workspace name in the top of the workspace window
Click on the current workspace name
Select a different workspace from the drop-down list

E. Rename Workspace
Make sure the radio button next to the workspace name is not selected
Type over the existing workspace name in the dialog box on the toolbar

F. Remove Workspace
File → Delete Workspace
Click **OK** to remove current workspace

G. Close Workspace
There are 2 different ways to close out of a workspace:
1. **File → Close → Workspace**
2. Click the red "X" in the upper corner of the workspace window

Note: Closing the final workspace window will close out of the entire project.

VIII. Select Data

A. Select Libraries

This function allows the user to select subsets of libraries via library names (or portions of library names), metadata, and/or specific OTUs. Library selection is often used in conjunction with workspaces to enable analysis of subsets of data within a project. A workspace may have ONE current filter; to create a new subset, a new workspace with a different current filter must be created.

Data → Select Libraries
New pop-up window appears for creation of filters
Click **New** on far right side of window
Enter desired filter name when prompted
Click **OK**
Filter name will appear in upper left corner of window

The popup window has three panes. The upper pane is for creating filters based on metadata and library names (column filter), and the middle pane is for selecting OTUs (row filter)...

1. Create a Library or Metadata Filter (Column Filter)
Click **Add** on right side of upper pane
Use the first pull-down menu to select metadata to filter by
Use the second pull-down menu to select filter operator
If one criterion does not include all the desired libraries, select **Add** again
· Another criterion can be added to the filter using the **And/Or** function.
To apply filter, click **Select** in upper right corner of window
· When a selection is made, the number of libraries and the library names included in the filter will be displayed at the bottom of the popup window.
Click **Save Filter** on far right side of window to keep the filter

2. Create an OTU Filter (Row Filter)
Click **Add** on right side of middle pane
Type in the OTU name you wish to filter by (any level of the hierarchy)
Use the first pull-down menu to select whether to filter by OTU name or member count
Use the second pull-down menu to select filter operator
Multiple OTUs can be filtered by using **Add** function
· Another criterion can be added to the filter using the **And/Or** function.
To apply filter, click **Select** in upper right corner of window
· When a selection is made, the number of libraries and the library names, and the number of hits in the selected OTU will be displayed at the bottom of the popup window.
Click **Save Filter** on far right side of window to keep the filter.

The way to think about how library selection and OTU selection work together is that FIRST a library is selected with the **Metadata Selection Criteria** rules, and then SECOND the OTUs for inclusion within a selected library are chosen with the **Taxonomy Selection Criteria** filter. In other words: OTU selection filters do not select or influence the selection of libraries; they only select the OTU values to be retained within libraries that are selected by the **Metadata Selection Criteria**.

B. Group Data

Explicet allows the data making up multiple libraries to be combined by averaging the relative abundances of included taxonomic categories. For example, one might want to compare all libraries obtained by sampling within a building (Group 1) with all libraries within the study resulting from outdoor samples (Group 2). Explicet assumes that a library is the set of results that represent all taxonomic results from a single sample. To combine libraries without introducing a bias associated with large differences in within-group library sample sizes, Explicet combines libraries by averaging the relative abundances for each OTU in each library. In Explicet, when grouped libraries are displayed as

"counts", the **Counts** displayed are computed backwards from the summed percent abundances for each taxon. Results for the OTUs, especially for those OTUs with small number of counts, or OTUs whose counts vary widely between the grouped libraries, may be higher or lower than one expects from knowing the raw sums of the counts that went into the groups. Users need to be aware of the statistical implications of any operations performed on grouped libraries, as the operations are performed on the averaged relative abundances, or imputed sequence counts, rather than the raw sequence counts.

1. Group Libraries

This function allows the user to assemble libraries into a larger category by shared metadata features (e.g., multiple libraries from the same patient or from multiple sites of the same chemistry).

Group → Group Libraries
A pop-up window will open
In left panel, select the metadata descriptor you wish to group by
Click **Add** button between panels
Name of metadata descriptor will appear in the right panel
- For metadata that are numbers, a bin size can be specified by clicking on the number in the **Bin Size** column and entering the desired size. For example, age metadata can be binned by 5, resulting in group names like 0-5, 5-10, 10-15.
Click **Group** at top right of window to accept grouping

In the main workspace, make sure the **Show Lib Groups** check box is selected. Columns should now have metadata groupings rather than library names.

a) Remove Metadata Group
If you wish to remove a metadata grouping, follow the same procedure, except select the undesired descriptor in the right panel and click **Remove** button between panels.

2. Group OTUs

Often it is desirable to use a more basal taxonomic category than that provided by the employed sequence classification software. This is easily done in Explicet by forming a new taxonomic category combining several individual categories. Explicet does not control the taxonomic categories that are combined, meaning any OTU name can be combined with any other OTU name (user beware). For instance, the group function can be used to create an arbitrary "Other" taxonomic category consisting of disparate OTUs. Grouping taxonomic categories is done within the OTU pane. Combining OTUs is performed by Explicet by summing the raw counts of all of the OTU lines selected for the group. **Group OTU** simply adds the raw counts together for each item in the group, UNLIKE **Group Libraries**.

There are two components to grouping OTU names: **OTU Groups** and **OTU Rule Sets**. Multiple OTU Groups, or a single OTU Group, may make up one OTU Rule Set. Every OTU Group must be part of an OTU Rule Set. Therefore, when creating a new OTU Group in a workspace, the user is required to create a new OTU Rule Set. Once an OTU Rule Set is established in a workspace, the user can add new OTU Groups or edit the existing ones in the current OTU Rule Set.

a) Create OTU Group and OTU Rule Set
Select the OTUs to combine
- Several OTUs can be selected by clicking the first OTU, then Shift-click on the last OTU.
- Non-sequential OTUs can be selected (or deselected) via Ctrl-click.
- Select a single OTU line to create a consistent grouping rule, such as grouping all OTUs that start with the same name.
Group → Group OTU
A new window appears
Enter desired **OTU Rule Set Name** and **Group Name** where prompted

- In a workspace which already has an active OTU Rule Set, the user cannot edit the OTU Rule Set Name. Adding a new group simply creates a new OTU Group within the current OTU Rule Set; click **Save** to save the new OTU Group to the current OTU Rule Set.
- Click **Save As** to make no changes to the original OTU Rule Set while *also* saving a copy of the current OTU Rule Set with the new OTU Group; if the user clicks **Save As**, a pop-up will appear for entering a new OTU Rule Set Name. The new OTU Rule Set becomes the OTU Rule Set assigned to the workspace. The name of the current OTU Rule Set assigned to the workspace is displayed in the title of the OTU pane on the workspace window.
- To create a consistent grouping rule, enter the rule under **Group All Taxonomies that Start with**. For example, to group all OTUs of the Bacteroidetes phylum, type Bacteria/Bacteroidetes under **Group All Taxonomies that Start with**.

Click **Save**

The pop-up will disappear

The group name appears alphabetically in the OTU table (its members removed from the table)

When OTUs are grouped, OTU names included in the group can be viewed by hovering your cursor over the group name.

OTU groupings are available in the **OTU Heatmap** function, but not in the **Pie Chart** function. Any and all OTU Groups that are created will be carried forward into the analyses performed, and graphics created, within the workspace in which the grouping was done.

Note: One item OTUs (such as OTU Group names or OTUs that can only be traced to kingdom level, like "Bacteria") are considered the last OTU position; single name OTUs will disappear when **OTU Show Last** is deselected.

For information on exporting OTU Rule Sets, see page 43.

b) Add Single OTU Name to Existing OTU Group

Select the OTU name to be added

Ctrl-click the group name in the OTU table

Group → Add OTU Names to OTU Group

The individual OTU name will be removed from the OTU table and added to the **OTU Group**

Note: While single OTU names can be added to an existing OTU Group, two or more OTU Groups cannot be grouped.

c) Remove OTU Group

Select the group name in the OTU table
- If nothing is selected, all groups in the OTU table will be ungrouped.

Group → Ungroup OTU

A new window appears
- Click **Save** to simply remove the OTU Group from the current OTU Rule Set.
- Click **Save As** to make no changes to the original OTU Rule Set while *also* saving a copy of the current OTU Rule Set without the selected OTU Group; if the user clicks **Save As**, a pop-up will appear for entering a new OTU Rule Set Name. The new OTU Rule Set becomes the OTU Rule Set assigned to the workspace. The name of the current OTU Rule Set assigned to the workspace is displayed in the title of the OTU pane on the workspace window.

Group name is removed from the OTU table; its members are re-added to the table

d) Remove Single OTU Name from Existing OTU Group

Select the group name in the OTU table

Group → Remove OTU Names from OTU Group

A pop-up will appear with a list of OTU names within the OTU Group
Select the OTU name to be removed
Click **Remove**
Click **OK**
The OTU name will be removed from the OTU Group and added back to the OTU table

e) Rename OTU Group
Select the group name in the OTU table
Group → Rename Group
Enter new **OTU Group Name** where prompted
Click **OK**

f) Edit OTU Rule Sets
Group → Edit OTU Rule Sets
A window will appear with a list of all available **OTU Rule Sets**
Select the **OTU Rule Set** of interest
- There are several different ways to view an OTU Rule Set definition:
  1. Click **View**
  2. Double-click on an OTU Rule Set name
  3. Ctrl-click the OTU pane title.
  4. To view an OTU Rule Set definition *and* its members: simply left-click the OTU pane title to view both the definition and a list of the OTU Group members within the workspace in which the title was clicked.
- Click **Merge** to join multiple OTU Rule Sets. Using Shift-click or Ctrl-click, the user can select two or more OTU Rule Sets from the OTU Rule Sets column, and then click **Merge**. A pop-up window will appear for creating a new name for the merged OTU Rule Set; OTU Rule Sets will be combined into a new OTU Rule Set, however, the original/individual OTU Rule Sets will still remain in the list.
- Click **Delete** to remove an OTU Rule Set from the project.
- Click **Rename** to change the name of an OTU Rule Set.
- Click **Save As** to keep the current OTU Rule Set and also create a copy of the OTU Rule Set (after prompting the user to enter a new OTU Rule Set name).

g) Workspace OTU Rule Set Manager
This function provides a simple way of managing which OTU Rule Sets are applied to which workspaces. The user can assign only one OTU Rule Set to each workspace, but multiple workspaces can have the same OTU Rule Set.

Group → Workspace OTU Rule Set Manager
A window will appear with two columns
- The list of workspaces, on the left, is static and un-editable. The column of OTU Rule Sets, on the right, consists of drop-down selectors with all possible OTU Rule Set options next to each workspace.
Adjust which **OTU Rule Sets** are applied to each **Workspace** as desired
Click **OK**

IX. Import/Export Workspace Rules

A. Filters

Instead of creating filters from scratch in each project or workspace, importing/exporting filters allows the user to share filters between projects.

1. Import

By importing filters, filter rules created in a different project can be transferred to a new project, preventing the user from needing to create new filters with the individual rules from scratch.

File → Import → Filters
Select file, click **Open**

The filters now exist in the available filters list. For more information on how to create and select filters, see page 39, "Select Libraries".

2. Export

To export filters, first create filters with the conditions exactly as you wish you export. This function exports all filters in the project.

File → Export → Filters
Enter desired filter output file name and location
Click **Save**

B. OTU Rule Sets

Re-creating a desired grouping for every new project can be a tedious process. Importing and exporting OTU Rule Sets automates the preservation and transfer of OTU groupings from project to project.

1. Import

By importing OTU Rule Sets, the specifications used to create OTU groups in a different project can be transferred and applied to a new project.

File → Import → OTU Rule Sets
Select file, click **Open**

The OTU Rule Sets now apply to the current project, and the OTU Groups will appear in the OTU table, grouped accordingly. For more information on how to remove OTU Groups, see page 41, "Remove OTU Group".

2. Export

To export OTU Rule Sets, first create OTU groups (for information on how to create OTU groups, see page 40, "Create OTU Group and OTU Rule Set") with the conditions exactly as you wish to export.

File → Export → OTU Rule Sets
Select the desired OTU Rule Sets
· To export all OTU Rule Sets, click OK without selecting any. Or, click Ctrl-a to easily select all OTU Rule Sets before clicking OK.
Enter desired filter output file name and location
Click **Save**

X. Export Data

On occasion it may be useful to export a hierarchy or OTU table for further analysis in a dedicated statistical software package such as SAS or R. To do so, display your data on the Explicet workspace exactly as you wish it to appear in the exported spreadsheet (i.e., check library groupings, OTU Start, OTU Width, OTU Last functions, etc.), then export to a .txt (tab-separated-value) formatted file. The text file can be imported into a variety of programs, including Microsoft Excel, SPSS, R, SAS, JMP, GraphPad, SigmaPlot, etc.

A. Hierarchy Table
File → Export → Hierarchy
Enter desired hierarchy output file name and location
Click **Save**

B. OTU Table
File → Export → OTU
A new pop-up window will appear
Choose the export format
- Select **Entire OTU Name** to export the full OTU name, regardless of the current display format.
- Select **Edited OTU Name** to export the abbreviated OTU name exactly as it is displayed in the current workspace.
- Choose the **OTU Name delimiter** (character(s) placed between columns); delimiter may be up to 6 characters, enter "tab" for tab.
- To format the export so that it is compatible for use with the program, R, select **Export to R**.

Click **OK**
Another pop-up window will appear
Enter desired OTU export file name and location
Click **Save**

XI. Figures

A. Pie Charts

Tools → Plot → Pie Chart
A new window will appear with the hierarchical data available in the workspace
Use the drop down arrows to the left of each taxonomic level to find the OTU of interest
Select the name of the OTU, click **Add to Pie** (upper right of window)

- Adjust the display mode using the options in the upper toolbar. See "Display" (page 33) for more information.
- To create sub-wedges in the pie chart (e.g., showing sub-taxa within a phyla), select both the phyla and the taxa of interest.
- The pie chart is composed only of the groups selected by the user. Therefore, it may not add to 100% unless all the groups are selected. If it is desired that the pie chart sums to 100%, select the taxa which are domain level 1 (e.g., "Bacteria") and any desired phyla, genera, etc.
- Often the minor components of the dataset are not desired in the pie chart, but the user will want to show these as a collapsed "Other" wedge. To do this, select all of the taxa that you want to collapse together, then click **Add to Other**. A group named "Other" will appear in the table in bold font. Note: All selected taxa must belong to the same parent-level of taxonomy (i.e., they must all share some common higher level of taxonomy). When you click **Add to Other**, the selected taxa are automatically added to the pie. Taxa included in collapsed "Other" wedge can be viewed by hovering your cursor over "Other". To remove the "Other" wedge, select "Other" and click **Remove from Pie** (the taxa is no longer bold and will return to the initial individual items).

The taxa changes to a bold font

- To remove data, simply select a bold font group, click **Remove from Pie**.

Once all the desired taxa are selected, click **Plot**
A new window will appear containing pie chart display options

- To display a single pie chart which includes all libraries' data, select the **Total Libraries** checkbox
- To view a pie chart which includes only one specific library's data, select the library name in the column
- To display a single pie chart which combines data only from the specific libraries selected in the column, select **Libraries on Plot**
- Up to 16 pie charts (totaled from Total Libraries, Libraries on Plot, and individual libraries) may be created at once
- Use the **Rows** and **Columns** options at the top of the window to adjust the display format

Click **OK** to view the pie charts

- To add/remove taxa, click **Done** (upper right corner of pie chart window) to return to the selection table.
- To change the display options, click **Data** (lower right side of pie chart window).

1. Plot Attributes

After generating a plot with the default settings, the user can change the appearance of the plot through the **Plot Attributes** button on the lower right side of the **Plot Results** window.

a) Titles / Axes

Click **Titles / Axes** tab within **Plot Attributes** pop-up window

- Enter a single title for multi-pie charts
- Sorted libraries have one or more metadata tag(s) pre-appended before the library name, separated by a colon. The libraries are then displayed alphabetically first by metadata tag(s), then by library name. If **Show Library Name** is deselected, the library names after the colon will disappear, but the metadata tag(s) will remain. Note: If libraries are not sorted and do not have metadata tag(s), then deselecting **Show Library Name** will *not*

drop the library name (to prevent blank axis labels). For more information on sorting libraries, see page 34, "Sort Libraries".

When finished, click **Apply** in upper right corner of window

b) Colors

Click **Colors** tab within **Plot Attributes** pop-up window
- To pick a different background color, click the "**…**" button and select a new color from the pop-up display.
- To pick a different wedge color, click on the color and select a new color from the pop-up display.

When finished, click **Save** in upper right corner of window

c) Legend and Labels

Click **Pie Chart** tab within **Plot Attributes** pop-up window
- Adjust whether **Value**, **Percentage**, and/or **Name** label the colors represented in the legend
- Adjust the legend **Separator** if desired
- Adjust the **Legend Location** if desired
- Adjust whether **Value**, **Percentage**, and/or **Name** label the wedges on the pie chart
- Adjust the pie chart label **Separator** if desired
- Adjust the pie chart **Label Location** if desired

When finished, click **Save** in upper right corner of window

d) Size

Click **Size** tab within **Plot Attributes** pop-up window
Use the sliders on each axis to adjust the size of the plot as necessary
- Note: Does not scale font, only graphics and spacing.

Click **Save** to return to the **Plot Results** window

B. Heatmaps

Tools → Plot → OTU Heatmap
A new window will appear with the OTU data table
To limit the number of OTUs displayed in the heatmap:
- Select entire dataset by simply clicking **Select Range** without defining any limiters
- Manual selection: click on desired OTUs
- Select range using limiting boxes on toolbar, then click **Select Range**: In the first box, type the lower bounding limit. In the second box, type the upper bounding limit. Note: The bounding numbers that are selected in the two boxes are dependent upon the current display mode; so keep in mind that if the display mode is in **Counts**, the bounding numbers will include those OTUs with corresponding *counts/integers*. However, if the display mode is in **% of Total**, the bounding numbers will include those OTUs with corresponding *percentages* of the total dataset.

Click **Plot** on lower right side of window
A new window will appear containing a list of the individual libraries
- To display a heatmap which includes all libraries' data, make sure all libraries are selected.
- To view a heatmap which includes only one specific library's data, select the single library name in the column.
- To view a combination of a few libraries, Ctrl-click the individual library names.

Click **OK** to view the heatmap
- To add/remove taxa, click **Done** (upper right corner of heatmap window) to return to the selection table.
- To change the included libraries, click **Data** (lower right side of heatmap window).

1. Plot Attributes

After generating a plot with the default settings, the user can change the appearance of the plot through the **Plot Attributes** button on the lower right side of the **Plot Results** window.

a) Titles / Axes
Click **Titles / Axes** tab within **Plot Attributes** pop-up window
- Enter desired plot and axis titles into the designated spaces
- Sorted libraries have one or more metadata tag(s) pre-appended before the library name, separated by a colon. The libraries are then displayed alphabetically first by metadata tag(s), then by library name. If **Show Library Name** is deselected, the library names after the colon will disappear, but the metadata tag(s) will remain. Note: If libraries are not sorted and do not have metadata tag(s), then deselecting **Show Library Name** will *not* drop the library name (to prevent blank axis labels). For more information on sorting libraries, see page 34, "Sort Libraries".
- Adjust axis label rotations as desired

When finished, click **Save** in upper right corner of window

b) Colors
Click **Colors** tab within **Plot Attributes** pop-up window
- To pick a different color spectrum, click **Change Colors** and select a new spectrum from the pop-up display.
- Setting the number of color levels is useful when defining a new color spectrum. To add new colors, start with any existing color spectrum, set the number of colors you want to have in the spectrum (**Color Levels**), then click on each color to change it to the desired color. Color spectrums can have anywhere between 2 and 8 colors blending together.
- To reverse the colors, click **Flip Colors.**

When finished, click **Save** in upper right corner of window

c) Values and Color Bar
Click **Heatmap** tab within **Plot Attributes** pop-up window
- Deselect **Display Heatmap Values** to remove the values from the display; specify number of **Decimal Places** in displayed heatmap values
- Enter desired color bar title
- Deselect the **Autoscale** option if desired; change color bar **Min/Max**

When finished, click **Save** in upper right corner of window

d) Size
Click **Size** tab within **Plot Attributes** pop-up window
Use the sliders on each axis to adjust the size of the plot as necessary
- Note: Does not scale font, only graphics and spacing.

Click **Save** to return to the **Plot Results** window

C. Stacked Bar Charts
Tools → Plot → OTU Stacked Bar
A new window will appear with the OTU data table
To limit the number of OTUs displayed in the chart:
- Select entire dataset by simply clicking **Select Range** without defining any limiters
- Manual selection: click on desired OTUs
- Select range using limiting boxes on toolbar, then click **Select Range**: In the first box, type the lower bounding limit. In the second box, type the upper bounding limit. Note: The bounding numbers that are selected in the two boxes are dependent upon the current display mode; so keep in mind that if the display mode is in **Counts**, the bounding numbers will include those OTUs with corresponding *counts/integers*. However, if the display mode is in **% of Total**, the bounding numbers will include those OTUs with corresponding *percentages* of the total dataset.

Click **Plot** on lower right side of window
A new window will appear containing a list of **Stacked Bar Modes** and the individual libraries
- Select **% of Selected** to display only the selected data and create bars of 100% based on the relative percentages of the selected OTUs in each bar.

- Select **% of Total** to create an "other" category from the non-selected OTUs and create bars of 100% based on the selected OTUs plus "other" category.
- Select **Counts** to display raw data counts in integers. This mode does not force the bars to 100%.
- To display a bar chart which includes all libraries' data, make sure all libraries are selected.
- To view a bar chart which includes only one specific library's data, select the single library name in the column.
- To view a combination of a few libraries, Ctrl-click the individual library names.

Click **OK** to view the bar chart
- To add/remove taxa, click **Done** (upper right corner of stacked bar window) to return to the selection table.
- To change the included libraries, click **Data** (lower right side of stacked bar window).

1. Plot Attributes

After generating a plot with the default settings, the user can change the appearance of the plot through the **Plot Attributes** button on the lower right side of the **Plot Results** window.

a) Titles / Axes

Click **Titles / Axes** tab within **Plot Attributes** pop-up window
- Enter desired plot and axis titles into the designated spaces
- Sorted libraries have one or more metadata tag(s) pre-appended before the library name, separated by a colon. The libraries are then displayed alphabetically first by metadata tag(s), then by library name. If **Show Library Name** is deselected, the library names after the colon will disappear, but the metadata tag(s) will remain. Note: If libraries are not sorted and do not have metadata tag(s), then deselecting **Show Library Name** will *not* drop the library name (to prevent blank axis labels). For more information on sorting libraries, see page 34, "Sort Libraries".
- Deselect the **Autoscale** option if desired; change axis **Min/Max/Step Size**
- Adjust axis label rotations as desired

When finished, click **Save** in upper right corner of window

b) Grid

Click **Grid** tab within **Plot Attributes** pop-up window
- Deselect **Show** to remove the gridlines from the display
- To change the gridline color, click the "**…**" button, and select a new color from the pop-up display
- To pick a different gridline pattern, select a new pattern from the **Pattern** dropdown menu

When finished, click **Save** in upper right corner of window

c) Colors

Click **Colors** tab within **Plot Attributes** pop-up window
- To pick a different background color, click the "**…**" button and select a new color from the pop-up display.
- To reverse the colors, click **Flip Colors**
- To pick a different bar color, click on the color and select a new color from the pop-up display.

When finished, click **Save** in upper right corner of window

d) Legend and Orientation

Click **Stacked Bar** tab within **Plot Attributes** pop-up window
- Adjust the orientation of the stacked bars: **Horizontal Bars** or **Vertical Bars**
- Adjust whether the bars are stacked **OTU Names** or stacked **Libraries**
- Adjust the **Gap Width** between each stacked bar if desired (set to 0% to make the bars touch)
- Adjust the **Legend Location** if desired

When finished, click **Save** in upper right corner of window

e) Size
Click **Size** tab within **Plot Attributes** pop-up window
Use the sliders on each axis to adjust the size of the plot as necessary
· Note: Does not scale font, only graphics and spacing.
Click **Save** to return to the **Plot Results** window

D. Manhattan Plots
These are the plots produced by the **Two-Part** test, **Two-Proportions** test, and **Wilcoxon** test.

1. Plot Attributes
After generating a plot with the default settings, the user can change the appearance of the plot through the **Plot Attributes** button on the lower right side of the **Plot Results** window.

a) Titles / Axes
Click **Titles / Axes** tab within **Plot Attributes** pop-up window
· Enter desired plot and axis titles into the designated spaces
· Sorted libraries have one or more metadata tag(s) pre-appended before the library name, separated by a colon. The libraries are then displayed alphabetically first by metadata tag(s), then by library name. If **Show Library Name** is deselected, the library names after the colon will disappear, but the metadata tag(s) will remain. Note: If libraries are not sorted and do not have metadata tag(s), then deselecting **Show Library Name** will *not* drop the library name (to prevent blank axis labels). For more information on sorting libraries, see page 34, "Sort Libraries".
· Adjust whether the display is **Linear** or **Log**
· Deselect the **Autoscale** option for each axis if desired; change axis **Min/Max/Step Size**
· Adjust axis label rotations as desired
When finished, click **Save** in upper right corner of window

b) Grid
Click **Grid** tab within **Plot Attributes** pop-up window
· Deselect **Show** to remove the gridlines from the display
· To change the gridline color, click the "**…**" button, and select a new color from the pop-up display
· To pick a different gridline pattern, select a new pattern from the **Pattern** dropdown menu
When finished, click **Save** in upper right corner of window

c) Colors
Click **Colors** tab within **Plot Attributes** pop-up window
· To pick a different background color, click the "**…**" button and select a new color from the pop-up display.
· To pick a different curve color, click on the color and select a new color from the pop-up display.
When finished, click **Save** in upper right corner of window

d) Legend and Labels
Click **Curves** tab within **Plot Attributes** pop-up window
· Adjust the **Legend Location** if desired
· Adjust the **Curve Width** if desired
· Adjust the **Line Marker Width** if desired
· Adjust the marker **Symbol** if desired
· Select **Display error bars** if desired
· Deselect **Display marker at rarefaction point** to remove the line markers from the display
When finished, click **Save** in upper right corner of window

e) Size

Click **Size** tab within **Plot Attributes** pop-up window

Use the sliders on each axis to adjust the size of the plot as necessary

· Note: Does not scale font, only graphics and spacing.

Click **Save** to return to the **Plot Results** window

E. Print

To print a plot, click **Print** on the lower right side of the **Plot Results** window

A print preview window with more options will appear

· The portrait and landscape icons/buttons at the top of the dialog can be toggled.

· Use the **Page Size** dropdown to choose from **Letter**, **A1** or **Custom**. When you first change a page size to **A1**, Explicet switches to millimeter **Units**, but you can switch back to inches if you so desire. The opposite is true for a page size of **Letter**.

· When **Paper Size** is changed, Explicet does not change the **Image Size** (this may mean that when switching between landscape and portrait, part of the image is off the page). Pull the slider to a smaller value to force it to fit on the page (click **Maintain Aspect Ratio** to move both sliders together). This was an intentional design decision so that the image size isn't disrupted, and the user can make the image any size they want.

· The **Change Page Size** button is greyed out unless you have it set to a custom page size and have changed one of the values.

· **Image Size**, **Page Size**, **Units**, and orientation are all saved with the figure; the user can save different sizes for exporting versus printing.

Click **OK** to keep changes

Click **Print**

Choose a printer and any desired printer settings, then click **Print**

F. Export

Explicet can export graphics images in either bit mapped (.bmp) or vector (.ps and .pdf) formats.

Vector formats can be rescaled (made larger or smaller) to any size with software tools like Adobe Illustrator or GIMP (GNU Image Manipulation Program). For simple display at the original size in Microsoft Word or PowerPoint, the .bmp format is fine. For significant changes in aspect ratio, such as when creating an image for a small figure in a publication, Illustrator/GIMP will likely be needed to maintain the relative proportionality of all of the elements of a graphic.

Note: Explicet's scaling capability in **Print** preview is limited to scaling the graphical elements only; the font size is fixed. Illustrator/GIMP is required when both graphical elements and font size need to change in concert.

1. Spreadsheets (Graphics Setup)

The data that form the basis for any graphic can be exported as a tab-delimited (.txt) file.

Click **Export** on lower right side of the graphic *setup* window

A new pop-up window will appear

Choose the export format

· Select **Entire OTU Name** to export the full OTU name, regardless of the current display format.

· Select **Edited OTU Name** to export the abbreviated OTU name exactly as it is displayed in the current workspace.

· Choose the **OTU Name delimiter** (character(s) placed between columns); delimiter may be up to 6 characters, enter "tab" for tab.

· To format the export so that it is compatible for use with the program, R, select **Export to R**.

Click **OK**

Another pop-up window will appear

Enter the desired export file name and location

Click **Save**

2. Graphics (Plot Results)

Graphics output by Explicet may be in either bit-map formats (such as .png) or vector formats (.pdf), which are readily imported by tools such as Microsoft Word, PowerPoint, and Adobe Illustrator.

Click **Export** in lower right corner of **Plot Results** window
A preview window with more options will appear

- · The portrait and landscape icons/buttons at the top of the dialog can be toggled.
- · Use the **Page Size** dropdown to choose from **Letter**, **A1** or **Custom**. When you first change a page size to **A1**, Explicet switches to millimeter **Units**, but you can switch back to inches if you so desire. The opposite is true for a page size of **Letter**.
- · The **Custom** size was specifically implemented to handle the case where default paper-based page sizes forced axes labels to superimpose during PDF generation.
- · When **Paper Size** is changed, Explicet does not change the **Image Size** (this may mean that when switching between landscape and portrait, part of the image is off the page). Pull the slider to a smaller value to force it to fit on the page (click **Maintain Aspect Ratio** to move both sliders together). This was an intentional design decision so that the image size isn't disrupted, and the user can make the image any size they want.
- · The **Change Page Size** button is greyed out unless you have it set to a custom page size and have changed one of the values.
- · **Image Size**, **Page Size**, **Units**, and orientation are all saved with the figure; the user can save different sizes for exporting versus printing.

Click **OK** to keep changes
Click **Export**
Enter the desired export file name and location
Select the type of file you wish to export (.pdf, .ps, or image options)
Click **Save**

G. Save as Figure

Similar to workspaces, figures are saved within the larger project, so they stay linked to the data from which they were created and do not create additional files on your computer.

Click **Done** in **Plot Results** window
Click **Save as Figure** in lower right corner of plot setup window
Enter the desired figure name
Click **OK**

1. Open Existing Figure

There are 2 different ways to open a figure:
1. **Tools → Figures**
2. Click the **Figures** button in the upper right corner of the current workspace window.

A pop-up window will open with the list of existing figures and associated specs. The column headings can be clicked to re-sort the list of figures according to **Plot Type**, **Filter**, **Workspace**, date **Created**, etc. From this window, figures can be opened, deleted, or renamed. Double-clicking on a figure name immediately opens the figure in its original workspace. By clicking **Open in Different Workspace**, the user can open a figure in a workspace other than the one in which it was created; opening a figure in a different workspace keeps both the original figure in the original workspace and creates a new figure in the newly selected workspace.

XII. Statistics

For more comprehensive information on ecological statistics please see Magurran [6] and Colwell [3].

A. Alpha Diversity

The alpha diversity metrics address species diversity of Explicet libraries. When comparing alpha diversity metrics between libraries of different sizes (different numbers of total counts per library), rarefaction is essential. Rarefaction is a process that randomly resamples all libraries to the sampling depth of the smallest library (called the rarefaction point), thus allowing the alpha diversity metrics to be compared directly. The curves generated by plotting the alpha diversity metrics versus sample size are called collectors curves and are indicative of how well-sampled libraries are. Species observed (**Sobs**) is a metric which provides a good intuitive example of how to interpret collector's curves. If a **Sobs** graph has a steep upwards slope at the terminal end of the curve, it indicates that additional taxa are likely to be found with a small amount of additional sampling effort. If the **Sobs** curve flattens out, a sufficient number of samples have likely been taken as no more taxa will be found with additional sequencing.

Tools → Analyze → Alpha Diversity
A new pop-up window will appear

1. Settings

a) Libraries

The user can select whether to calculate diversity for all the libraries, a subset of libraries, or groups of libraries by clicking on the various options at the top and right of the pop-up window. For descriptions of these options, see page 33, "Display".

b) Bootstrap

Bootstrap is a way to estimate standard error for complex parameters and a way to check the constancy of results. It sets the number of iterations that a set of calculations will be repeated for alpha diversity estimation. Bootstrap is the name of the process that Explicet uses to provide rarefaction for the alpha diversity metrics. Clicking on the **Bootstrap** button causes Explicet to start the random resampling process. At rarefaction, Bootstrap is resampled to the size of the smallest library.

Default: 25. The dataset will be resampled 25 times.

c) Cutoff Size

Varies by dataset. The default will select all libraries ≥ the 3rd sigma of the average library size value. Some very small libraries may be excluded, which will be displayed directly to the right of the **Cutoff Size** box.

d) # Steps

In generating a collector's curve, the step size controls the incremental increase in number of sequences between resampling events, in other words the number of data points in each curve. The number of sequences in the smallest included library is divided by **# Steps** to create the step size. This step size will then be applied to all the larger libraries for calculations and rarefaction. The number of sequences per step is displayed to the right of the **# Steps** box.

Default: 0 steps.
- By default, Explicet calculates a **Single statistic at Rarefaction point only**. To remove this default, deselect the checkbox below the **# Steps** box.
- When **# Steps** is set at the default (0 steps), then **Single statistic at Rarefaction point only** is *unchecked*, **# Steps** will automatically change to 10.

- · Note: As the **# Steps** increases, **Bootstrap** will take longer to run. To create smoother curves, increase **# Steps**, but keep in mind that the length of time to run **Bootstrap** will increase exponentially.

e) Use Min Lib Size

Using the minimum library size is necessary for comparison of multiple curves/libraries. Most studies will have this option checked; **Use Min Lib Size** should be checked to reduce bias. For example, if you have one library with 500 sequences and another library with 10,000 sequences, the value at rarefaction is where the *smallest* library size falls; this way, the libraries are compared at the same level of sampling effort.

You may choose to un-check **Use Min Lib Size** if you're looking at individual libraries irrespective of other libraries. Without **Use Min Lib Size** checked, sampling starts at each library's total **Counts** divided by 10 (when **# Steps**: 10). Note that if this option is not selected, rarefaction will not apply due to sampling variation/sampling bias between libraries.

f) User Modifications

The user may modify any of the default settings to be more or less stringent.

2. Calculate

After selecting all the appropriate settings, click **Bootstrap** on upper right side of window. This will generally take a few seconds to minutes depending on computer processing speed. You will be unable to use other Explicet functions while the program is calculating alpha diversity.

3. Tests

a) Sobs

The number of taxa/OTUs observed in a library.

b) Singletons

The number of taxa/OTUs observed exactly once in a library.

c) Doubletons

The number of taxa/OTUS observed exactly twice in a library.

d) ACE

Abundance based Coverage Estimator. The number of predicted taxa/OTUs based on observed singletons and rare taxa.

e) ACEVar

Coefficient of variation of the Abundance based Coverage Estimator.

f) Chao1

The number of species predicted based on observed singletons and doubletons.

g) Chao195ciL

Chao1 lower 95% confidence interval based on the bootstrap.

h) Chao195ciU

Chao1 upper 95% confidence interval based on the bootstrap.

i) Goods

A measure of how well the amount of sequencing done for a library represents the biodiversity in the library. Low coverage means that the library is under-sampled and may require additional sequencing. "Good's Coverage" is measured on a 0-100% scale, with 100% indicating that all expected OTUs have been observed.

j) ShannonH

Shannon diversity index, H (log base 2). This measure of biodiversity incorporates both OTU richness and OTU evenness into a single value. Greater OTU count and more uniform distribution of OTUs increase H.

k) ShannonE

Shannon evenness index, H/Hmax (log base 2). Measures the uniformity of a distribution of OTUs on a 0-100% scale. A perfectly uniform distribution will have E = 100%.

l) Simpson

Simpson's index, D: Another measure of complexity that measures the probability that two randomly selected individuals belong to the same OTU.

m) SimpsonD

Simpson's diversity index: 1 – D.

n) SimpsonE

Simpson's evenness index.

o) SimpsonR

The reciprocal of Simpson's index: 1/D.

4. Plot

After calculating the alpha diversity, click **Plot** on lower right side of window. A pop-up will appear with all the diversity measures and library names. Select the desired parameters, then click **OK** to plot the rarefaction curves for the selected measure and libraries. Toggle the display of individual curves on and off by clicking on the legend. Click **Remove Deselected Curves** to remove the curves from the figure. See page 45 for details on how to modify titles, axis labels, and colors.

B. Beta Diversity

1. Tests

a) Morisita-Horn

$$D_{Morisita-Horn} = 1 - 2\frac{\sum \frac{S_{A,i} S_{B,i}}{n \quad m}}{\sum \left(\frac{S_{A,i}}{n}\right)^2 + \sum \left(\frac{S_{B,i}}{m}\right)^2}$$

$S_{A,i}$ = the number of individuals from community A in the ith OTU
$S_{B,i}$ = the number of individuals from community B in the ith OTU
n = the number of individuals in community A
m = the number of individuals in community B

(Formula from Mothur website: http://www.mothur.org/wiki/Morisitahorn)

Morisita-Horn is a beta diversity measure on a 0-1 scale, indicating relative similarity between OTUs contained within two libraries; 1 = identical overlap in OTUs between libraries, 0 = no shared OTUs between two libraries.

Tools → Analyze → Beta Diversity → Morisita-Horn
A new window will appear that contains a comparison matrix with the calculated Morisita-Horn values for each pair of libraries or metadata groupings. A value of 1.000 indicates two groups are identical, which is why the diagonal of the matrix (libraries compared to themselves) has values of 1.000.

b) Bray-Curtis

$$D_{Bray-Curtis} = 1 - 2\frac{\sum min(S_{A,i}, S_{B,i})}{\sum S_{A,i} + \sum S_{B,i}}$$

$S_{A,i}$ = the number of individuals in the ith OTU of community A
$S_{B,i}$ = the number of individuals in the ith OTU of community B

(Formula from Mothur website: http://www.mothur.org/wiki/Braycurtis)

Bray-Curtis is a beta diversity measure on a 0-1 scale, indicating relative dissimilarity between OTUs contained within two libraries; 0 = identical overlap in OTUs between libraries, 1 = no shared OTUs between two libraries.

Tools → Analyze → Beta Diversity → Bray-Curtis
A new window will appear that contains a comparison matrix with the calculated Bray-Curtis values for each pair of libraries or metadata groupings. A value of 0.000 indicates two groups are identical, which is why the diagonal of the matrix (libraries compared to themselves) has values of 0.000.

c) ThetaYC

$$D_{\theta YC} = 1 - \frac{\sum_{i=1}^{S_T} a_i b_i}{\sum_{i=1}^{S_T}(a_i - b_i)^2 + \sum_{i=1}^{S_T} a_i b_i}$$

S_T = the total number of OTUs in communities A and B
a_i = the relative abundance of OTU *i* in community A
b_i = the relative abundance of OTU *i* in community B

(Formula from Mothur website: http://www.mothur.org/wiki/Thetayc)

ThetaYC is a beta diversity measure on a 0-1 scale, indicating relative dissimilarity between OTUs contained within two libraries; 0 = identical overlap in OTUs between libraries, 1 = no shared OTUs between two libraries.

Tools → Analyze → Beta Diversity → ThetaYC
A new window will appear that contains a comparison matrix with the calculated ThetaYC values for each pair of libraries or metadata groupings. A value of 0.000 indicates two groups are identical, which is why the diagonal of the matrix (libraries compared to themselves) has values of 0.000.

2. Display Options
The matrix display, and subsequent plots, can be modified by rearranging libraries to group by higher values (column total of each library) in either the upper left (**Descending by Value**) or lower right (**Ascending by Value**) corner. The default order is **Alphabetical by Library Name**, which can be useful if libraries within a metadata group are defined by similar names.

3. Plot
A heatmap of the matrix is generated by clicking **Plot** on the lower right side of window. See the section on heatmaps (page 46) for information on modifying the plot attributes.

C. Two-Part Test
This tool provides a statistical test of whether each OTU differs significantly between two groups of data. The Two-Part test is the sum of two test statistics, one comparing the proportion of non-zero counts and one comparing the medians of the non-zero counts. The statistical approaches incorporated in Explicet are useful for identification of taxa that differentiate two groups. The Two-Part statistic makes

use of a common practice in statistics by adding the Chi^2 statistics from two independent tests. This was developed to address the issue of inverse relationship between proportion of samples that contained a taxon and the median relative abundance of that taxon. For more information on the Two-Part test, please see: Wagner et al [11].

The input requires that the user select two mutually exclusive categories of libraries (e.g., healthy and diseased) using filters. To create filters see page 39, "Select Libraries". The developers recommend that at least five libraries be in each category so that the test is statistically valid.

Tools → Analyze → Two-Part

1. Settings

    a) Select Filters
The number of libraries included in each filter will be displayed immediately to the right of the filter selection. If no filters have been created, click **Setup Filters** on upper right side of window and proceed as described on page 39, "Select Libraries".

    b) Select P-Threshold
Default: 0, which will show the P-value for every OTU.

    c) OTU Display
Select the desired OTU name display options, see page 33 for details.

2. Output
The output table displays the following variables with values for each of the taxa: m1, p1, med1, m2, p2, med2, chi^2 (chi**2), PValue and -Log(PV)…

    a) m1 and m2
The number of samples with non-zero sequence counts for each category.

    b) p1 and p2
The % of non-zero counts for each corresponding group: p1 and p2 are the prevalences of an OTU in the two categories.

    c) med1 and med2
The median of the non-zero sequence counts (or median of the "m" samples).

    d) chi2**
The chi-squared test statistic for the two-part test.

    e) PValue
The corresponding parametric p-value from the two-part test.

    f) -Log(PV)
The negative of the log(base 10) of the p-value. This transformation is used to easily see those taxa which are statistically significant in the Manhattan plot.

3. Plot
Once Two-Part statistics have been calculated, the data can be visualized as a Manhattan plot (scatterplot with lines) by clicking **Plot** on lower right side of window. The x-axis is the OTU number from the Two-Part spreadsheet; the y-axis is the -Log(PV). The plots have three horizontal marker lines. The bottom line indicates results approaching significance at a p-value of 0.1. The middle line denotes significance at a p-value of 0.05, and the top line denotes significance at a p-value of 0.01.

See page 45 for details on how to modify titles, axis labels, and colors.

D. Two-Proportions Test

The Two-Proportions test identifies the differences in proportion between two populations. This comparative tool performs a continuity-adjusted chi-square test to evaluate the difference in the detection rates across the two groups for each taxa/OTU.

The input requires the user to apply two mutually exclusive filters to the libraries tested. To create filters see page 39, "Select Libraries".

Tools → Analyze → Two-Proportions

1. Settings

a) Select Filters
The number of libraries included in each filter will be displayed immediately to the right of the filter selection. If no filters have been created, click **Setup Filters** on upper right side of window and proceed as described on page 39, "Select Libraries".

b) Select P-Threshold
Default: 0, which will show the P-value for every OTU.

c) OTU Display
Select the desired OTU name display options, see page 33 for details.

2. Output
The output table displays the following variables with values for each of the taxa: m1, p1, m2, p2, Z, PValue and -Log(PV)…

a) m1 and m2
The number of samples with non-zero sequence counts for each corresponding group.

b) p1 and p2
The % of non-zero counts for each corresponding group.

c) Z
The two-proportions value.

d) PValue
The corresponding parametric p-value from the two-proportions test.

e) -Log(PV)
The negative of the log(base 10) of the p-value.

3. Plot
Once Two-Proportion statistics have been calculated, the data can be visualized as a Manhattan plot (scatterplot with lines) by clicking **Plot** on lower right side of window. The x-axis is the OTU number from the Two-Proportion spreadsheet; the y-axis is the -Log(PV). The plots have three horizontal marker lines. The bottom line indicates results approaching significance at a p-value of 0.1. The middle line denotes significance at a p-value of 0.05, and the top line denotes significance at a p-value of 0.01.

See page 45 for details on how to modify titles, axis labels, and colors.

E. Wilcoxon Test
The Wilcoxon test is a nonparametric statistical calculation used to compare the median OTU abundances of two categories. A non-parametric Wilcoxon test with a continuity correction is employed because OTU abundances are not necessarily normally distributed. This version of the Wilcoxon test compares the median relative abundance from all samples. This differs from the Wilcoxon test used in the Two-Part test, which includes only samples with median relative abundances greater than 0.0.

The input requires the user to apply two mutually exclusive filters to the libraries tested. To create filters see page 39, "Select Libraries".

Tools → Analyze → Wilcoxon

1. Settings

a) Select Filters
The number of libraries included in each filter will be displayed immediately to the right of the filter selection. If no filters have been created, click **Setup Filters** on upper right side of window and proceed as described on page 39.

b) Select P-Threshold
Default: 0, which will show the P-value for every OTU.

c) OTU Display
Select the desired OTU name display options, see page 33 for details.

2. Output
The output table displays the following variables with values for each of the taxa: m1, n1, p1, med1, m2, n2, p2, med2, W, PValue and -Log(PV)…

a) m1 and m2
The number of samples with non-zero sequence counts for each corresponding category.

b) n1 and n2
The total number of samples in each category.

c) p1 and p2
The % of non-zero counts for each corresponding category.

d) med1 and med2
The median of the non-zero sequence counts (or median of the "m" samples).

e) W
The Wilcoxon value.

f) PValue
The parametric p-value from the two-part test.

g) -Log(PV)
The negative of the log(base 10) of the p-value. This transformation provides a convenient means of plotting p-values, such as in a Manhattan plot.

3. Plot
Once Wilcoxon statistics have been calculated, the data can be visualized as a Manhattan plot (scatterplot with lines) by clicking **Plot** on lower right side of window. The x-axis is the OTU number from the Wilcoxon spreadsheet; the y-axis is the -Log(PV). The plots have three horizontal marker lines. The bottom line indicates results approaching significance at a p-value of 0.1. The middle line denotes significance at a p-value of 0.05, and the top line denotes significance at a p-value of 0.01.
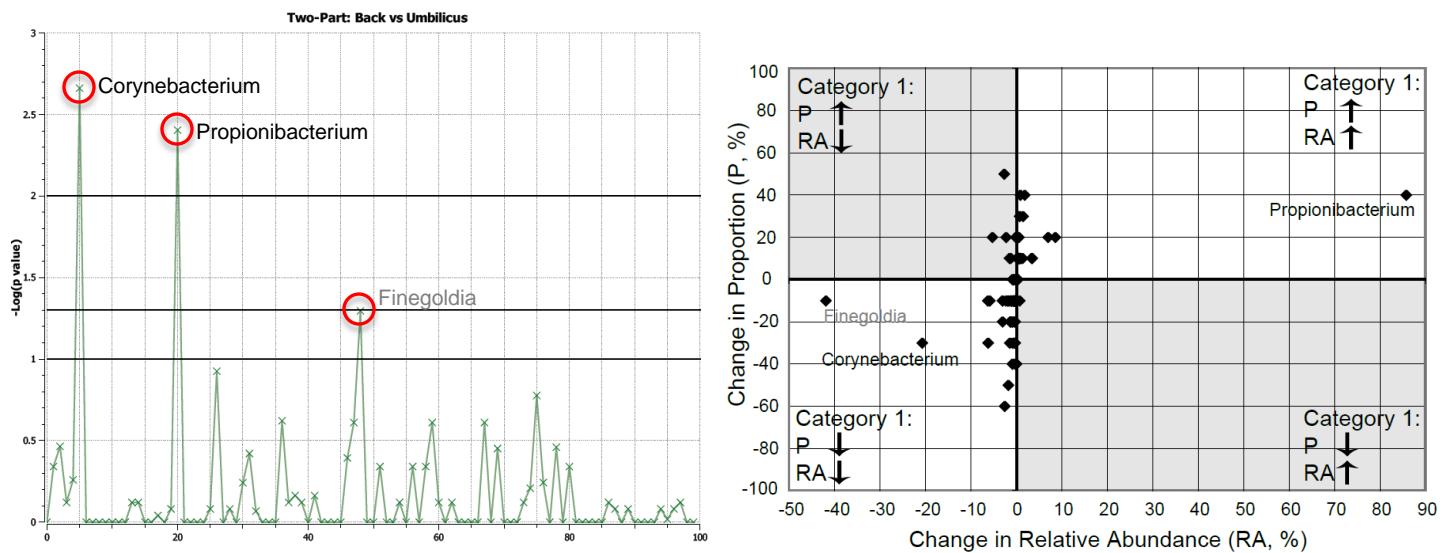
See page 45 for details on how to modify titles, axis labels, and colors.

XIII. Comparing Two-Part, Two-Proportions, Wilcoxon Tests

The following table documents under which conditions results for each of the comparative tests will be obtained.
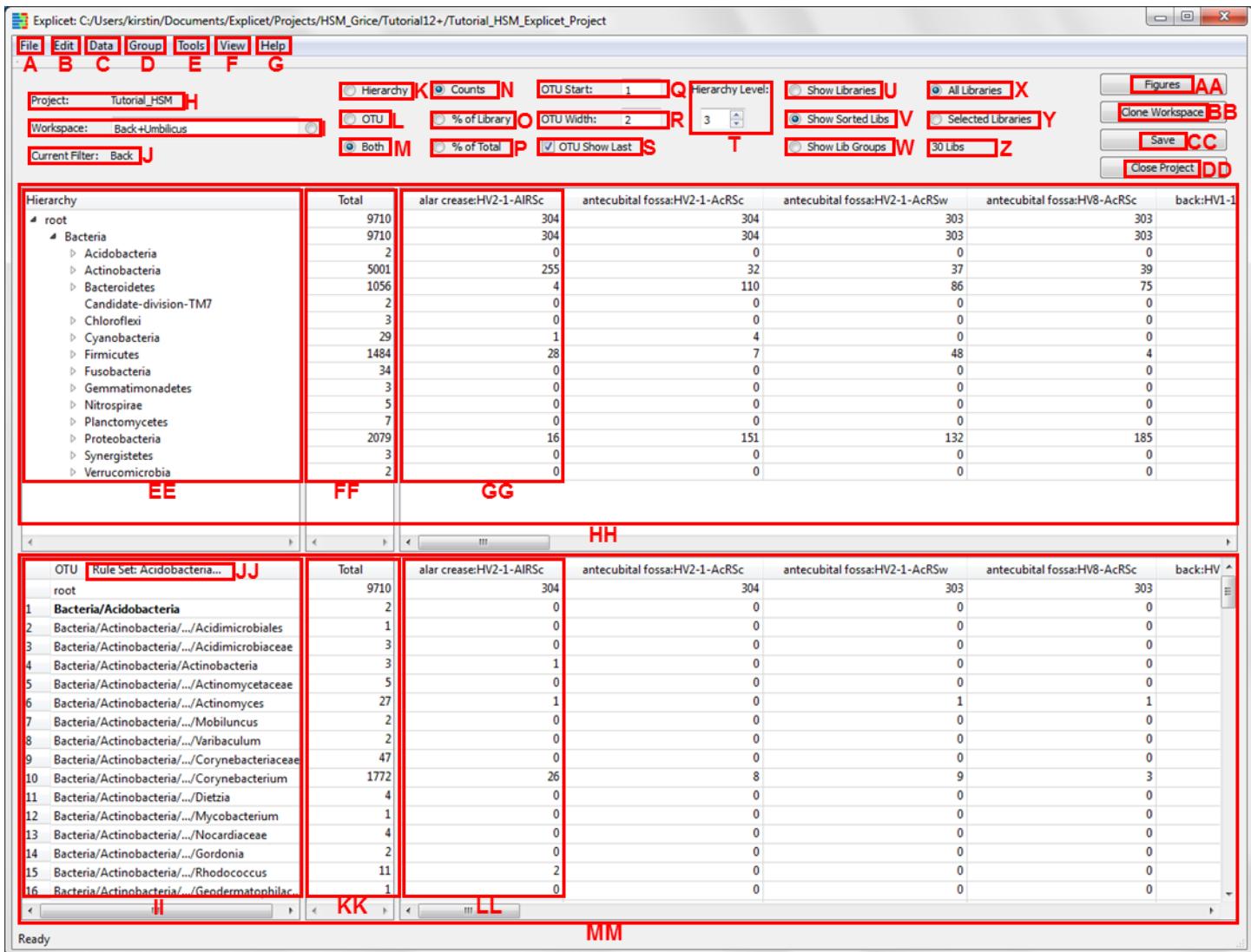
Conditions		Two-Part	Two-Proportions	Wilcoxon
Category 1	Category 2			
m1 = 0	m2 = 0	---	---	---
m1 ≤ 1	m2 > 1	✓	✓	---
m1 > 0	m2 ≤ 1	✓	✓	---
m1 >1	m2 > 1	✓	✓	✓

The following plot and chart demonstrate how to decide which of the three tests (Proportion, Wilcoxon, Two-Part) holds the most power for an individual dataset. For more information on how this plot was created, please view the Explicet Tutorial. The tutorial and associated data are obtainable from the website: www.explicet.org

The points farthest from the origin are labeled. The names in black were identified as significant; the points with grey text were not statistically significant. The two greyed out quadrants are situations in which the Two-Part test has power to identify significant differences (where the component statistics lose power due to the inverse relationship between proportion and relative abundance average out). While *Finegoldia* has a large change in relative abundance this is due to detection at ~41% relative abundance in a single umbilicus library. These data can be simplified by excluding taxa not present in a majority of libraries from either group. This graphic was generated by exporting the Two-Part test statistic data from Explicet and processing in Microsoft Excel.

Explicet Directory

Code	Label	Function
A	File	New: Project (pg. 36), Project from Current Project (pg. 36), Workspace (pg. 38), Workspace from Current Workspace (pg. 38) Open: Project (pg. 36), Workspace (pg. 38) Close: Project (pg. 36), Workspace (pg. 38) Save (pg. 36) Save As (pg. 36) Delete Workspace (pg. 38) Import: File (pg. 23), Directory (pg. 24), Metadata (pg. 30), Filters (pg. 43), OTU Rule Sets (pg. 43) Export: Hierarchy (pg. 44), OTU (pg. 44), Metadata (pg. 31), Filters (pg. 43), OTU Rule Sets (pg. 43) Exit (pg. 36)
B	Edit	Project Settings (pg. 36)
C	Data	Select Libraries (pg. 39) Metadata (pg. 30)
D	Group	Group Libraries (pg. 40) Group OTU (pg. 40) Ungroup OTU (pg. 41)

		Add OTU Names to OTU Group (pg. 41)
		Remove OTU Names from OTU Group (pg. 41)
		Rename OTU Group (pg. 42)
		Workspace / OTU Rule Set Manager (pg. 42)
		Edit OTU Rule Sets (pg. 42)
E	Tools	Analyze: Alpha Diversity (pg. 52), Beta Diversity (pg. 54), Two-Part (pg. 55), Two-Proportions (pg. 57), Wilcoxon (pg. 57) Plot: Pie Chart (pg. 45), OTU Heatmap (pg. 46), OTU Stacked Bar (pg. 47) Figures (pg. 51)
F	View	Sort Libraries (pg. 34) Unsort OTU (pg. 35)
G	Help	About
H	Project	displays current Project name (Projects, pg. 36)
I	Workspace	displays current Workspace name (Workspaces, pg. 38)
J	Current Filter	displays current Filter name (Filters, pg. 39)
K	Hierarchy	displays Hierarchy pane only (pg. 33)
L	OTU	displays OTU pane only (pg. 33)
M	Both	displays Hierarchy and OTU panes (pg. 33)
N	Counts	displays raw sequence data counts in integers (pg. 33)
O	% of Library	displays data normalized by column (pg. 33)
P	% of Total	displays data normalized to total dataset (pg. 33)
Q	OTU Start	taxonomic level to start lineage lines in OTU pane (pg. 33)
R	OTU Width	width of number of lineage levels selected (pg. 33)
S	OTU Show Last	appends last item in taxonomic line onto truncated OTU lineage (pg. 33)
T	Hierarchy Level	sets default number of taxonomic levels expanded in hierarchy pane (pg. 34)
U	Show Libraries	displays the libraries by name and sorts them alphabetically (pg. 34)
V	Show Sorted Libs	displays libraries alphabetically first by metadata tag, then by library name (pg. 34)
W	Show Lib Groups	displays combined OTU counts from all libraries within selected metadata group (pg. 34)
X	All Libraries	displays all libraries in the workspace based on sort/group selections (pg. 34)
Y	Selected Libraries	displays only the libraries included in the current filter (pg. 34)
Z	# Libraries	number of libraries displayed
AA	Figures	click to view the list of figures within the project (Figures, pg. 51)
BB	Close Workspace	click to close the workspace
CC	Save	click to save the project
DD	Close Project	click to close the project
EE	Hierarchy	data collapsed into taxa with triangular expansion buttons - clicking on an expansion button expands only the selected phyla to the next taxonomic level (pg. 33)
FF	Total	displays the total for each taxa in "EE" (Raw and Normalized Data Options, pg. 33)
GG	Library Name	displays each library and its individual total for each taxa in "EE"
HH	Hierarchy Pane	displays data collapsed into taxa with triangular expansion buttons (pg. 33)
II	OTU	displays each taxonomic classification listed individually, often resulting in very long OTU names (pg. 33)
JJ	OTU Rule Set	click to view the OTU Rule Set definition and its members (pg. 42)
KK	Total	displays the total for each taxa in "II" (Raw and Normalized Data Options, pg. 33)
LL	Library Name	displays each library and its individual total for each taxa in "II"
MM	OTU Pane	displays each taxonomic classification listed individually (pg. 33)

Acknowledgements

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