5. Run a PERMANOVA

Overview

If you have purchased a subscription to *PRIMER 8 with PERMANOVA+*, then you will have access to the **PERMANOVA+** main menu item that allows you to perform a broad range of additional analyses using a suite of routines that are not available in the basic *PRIMER 8 Lite* package. Check out our website to see a **Detailed list of all features**.

Note also that the **PERMANOVA** routine has been expanded substantially in the leap from PRIMER 7 to PRIMER 8. For a more complete guide to all of these important improvements, please see the essential resource: '**What's new in PRIMER 8**'. You can also consult the historical **PERMANOVA+ user manual** for fundamental information and references regarding some of these routines and their underlying statistical details.

Here, we will run through a quick example of how to set up and run a multi-factor PERMANOVA analysis in PRIMER 8. **Permutational multivariate analysis of variance** (PERMANOVA) partitions variation in the space of a chosen dissimilarity measure in response to one or more factors in a specified sampling protocol or experimental design (Anderson (2001a) , Anderson (2017)). Tests of individual terms in a PERMANOVA model are achieved by constructing correct (pseudo-)*F* ratios on the basis of expectations of mean squares (EMS), and p-values are obtained using correct permutation algorithms given the full study design. *We know of no other software package that accomplishes this.*

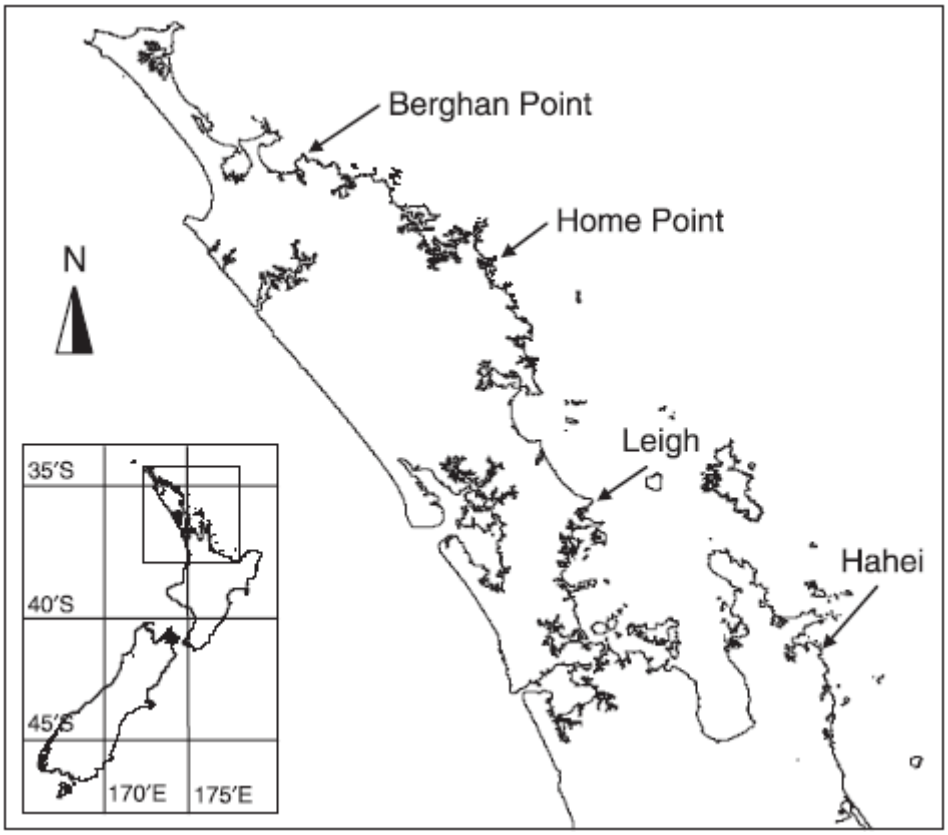
Importantly, the PERMANOVA routine in PRIMER allows the user:

- to specify whether factors are **fixed**, **random**, **finite**, or of a type called '**whole-plot/subject**' that caters to designs lacking replication at different scales,
- to account for **heterogeneity** in multivariate dispersions when testing for differences in centroids,
- to specify whether a factor is **nested** in one or more other factors,
- to test **interaction terms**,
- to include one or more quantitative **covariates** in the analysis,
- to **group covariates** (accommodating cyclical/seasonal models),
- to **re-order**, **pool** or **remove** individual terms from a model,
- to handle correctly:
  - **mixed models**
  - user-specified **contrasts**
  - **BACI designs** (before-after/control-impact),
  - **asymmetrical designs** (e.g., in environmental impact studies),
  - **randomised blocks**,
  - **split plots** and **splt-split-plots**,
  - **hierarchical designs**,
  - **repeated measures**,
  - **unbalanced designs** (Type I, II or III sums of squares),

- ... and more.

A three-factor hierarchical design

We will run PERMANOVA on an example dataset consisting of assemblages of molluscs collected from holdfasts of the kelp *Ecklonia radiata* in a 3-factor hierarchical experimental design. There were *n* = 5 holdfasts collected from each of 2 areas (tens of meters apart) at each of 2 sites (hundreds of meters to kilometers apart) from each of 4 locations (hundreds of kilometers apart) in rocky reef habitats along the northeastern coast of New Zealand (Anderson *et al.* (2005a) , Anderson *et al.* (2005b)).



The map above shows the four locations on the northeastern coast of New Zealand from which holdfasts were collected for the study: Berghan Point, Home Point, Leigh and Hahei (reproduced from Fig. 1 in Anderson et al. (2005a)).

The example data are found in the file called 'NE_NZ_holdfast_fauna.pri' in the folder named ' NE_NZ_holdfasts' inside the 'Examples_P8' folder that can be downloaded by clicking **Help** > **Get Examples...**. There were 351 taxa (rows) from 15 different phyla quantified in this study. Here, we shall focus only on the phylum Mollusca (105 taxa).

Our interest lies in quantifying the degree of turnover in the identities of mollusc species at different spatial scales, as measured by the Jaccard resemblance measure. This a fully hierarchical sampling design with three spatial factors, as follows:

- Locations (random with 4 levels: Berghan Point, Home Point, Leigh and Hahei)
- Sites (random and nested in Locations, with 2 sites per location)

- Areas (random and nested in Sites, with 2 areas per site)

Areas are therefore also (necessarily) nested in Locations as well.

Steps in a PERMANOVA analysis

The two essential steps required to run a PERMANOVA analysis in PRIMER are always:

- *first*, **specify the design**; and
- *second*, **run the PERMANOVA analysis**, given the design, on a chosen resemblance matrix (arising from the data of interest).

Generally, we first need to get our data in to PRIMER, perform appropriate pre-treatment(s), if any, then calculate a resemblance matrix from this. A resemblance matrix will always serve as the starting point for any PERMANOVA analysis.

For this example...

An analysis of only the mollusc species from the holdfasts in accordance with the three-factor hierarchical design, based on Jaccard resemblances, can be done by following these steps:

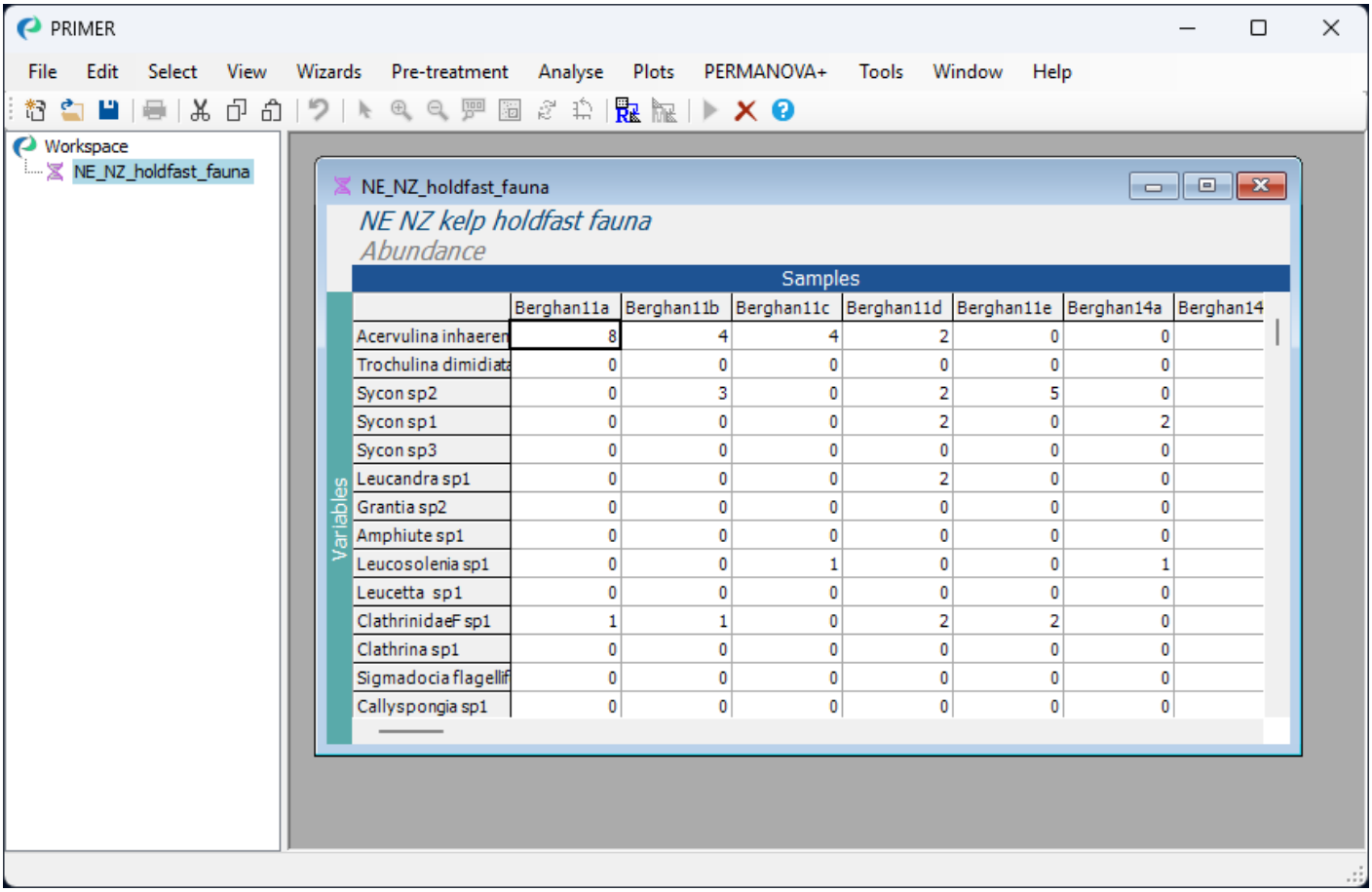
1. **Open the example data file** in the PRIMER workspace and **select a subset of the variables** corresponding to only the mollusc species for what follows.
2. Calculate **Jaccard similarities*** among the sampling units (individual holdfasts).
3. **Specify the experimental/sampling design** (creating a design file).
4. **Run the PERMANOVA routine** (to obtain a partitioning and p-values *via* permutation for each term in the model.
5. Do an **ordination of distances among centroids** to visualise the effects and the relative importance of the factors.

*(*Note: the Jaccard resemblance measure utilises only presence/absence information, so we do not need to perform a pre-treatment transformation step for this example.)*

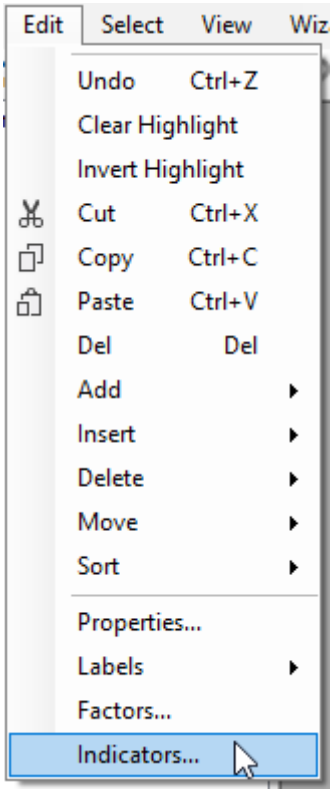
Step 1: Data selection

Open up the example data file

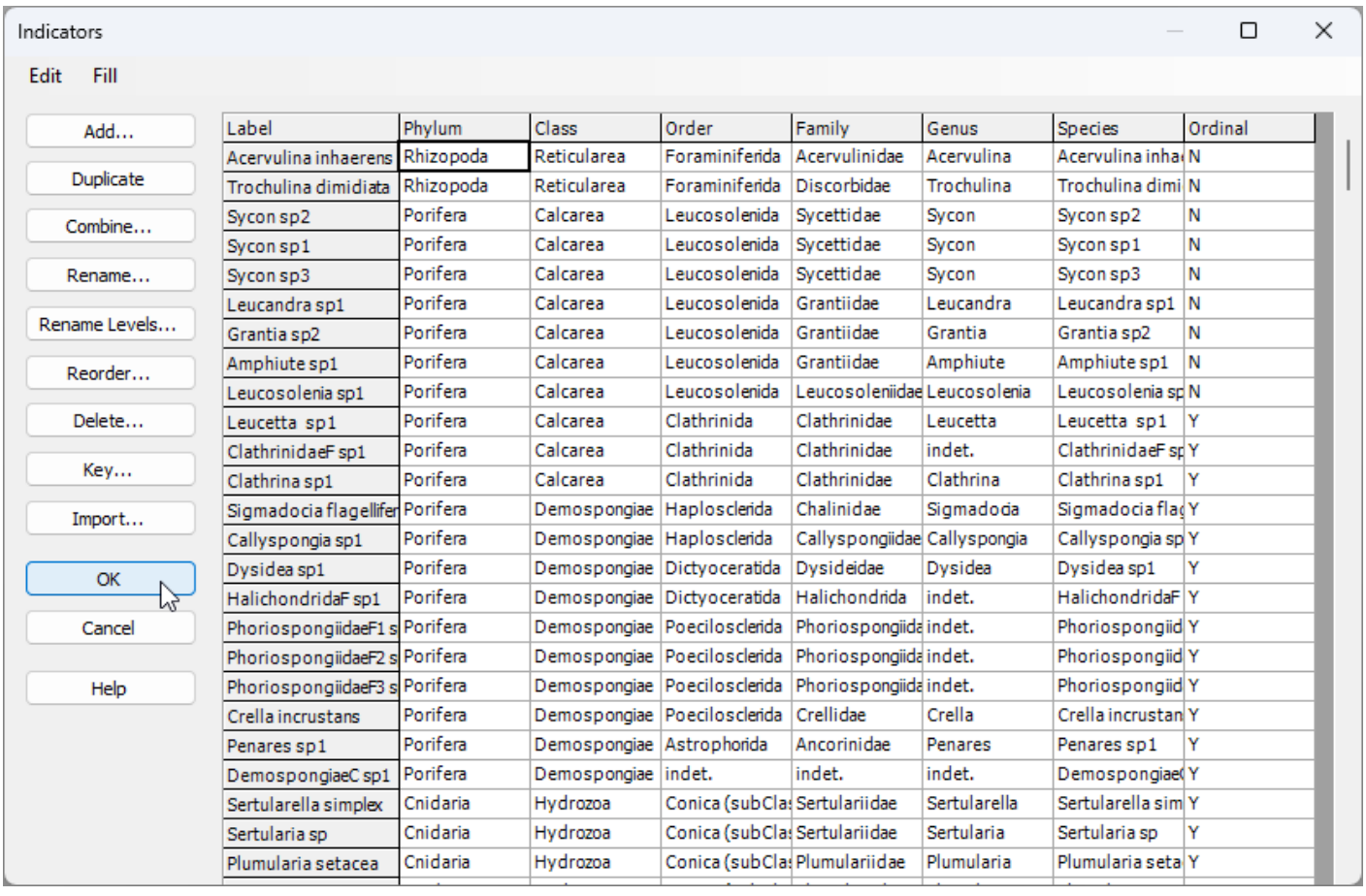
Launch PRIMER, then click **File** > **Open...** from the main menu, navigate to the folder named 'NE_NZ_holdfasts' in the 'Examples_P8' directory, and select 'NE_NZ_holdfast_fauna.pri'. Click **Open** to display the species matrix.



Click on **Edit** > **Indicators...**



You will see that there are indicators for the variables in this datasheet that show the taxonomic groups in which each species (or taxon) variable belongs, with different levels of the taxonomic hierarchy being provided as different indicators (i.e., 'Genus', 'Family', 'Order', 'Class' and 'Phylum'). There is also an indicator to identify whether individual taxa were counted (enumerated) or quantified on an ordinal scale ('Ordinal' = 'N' or 'Y', respectively).



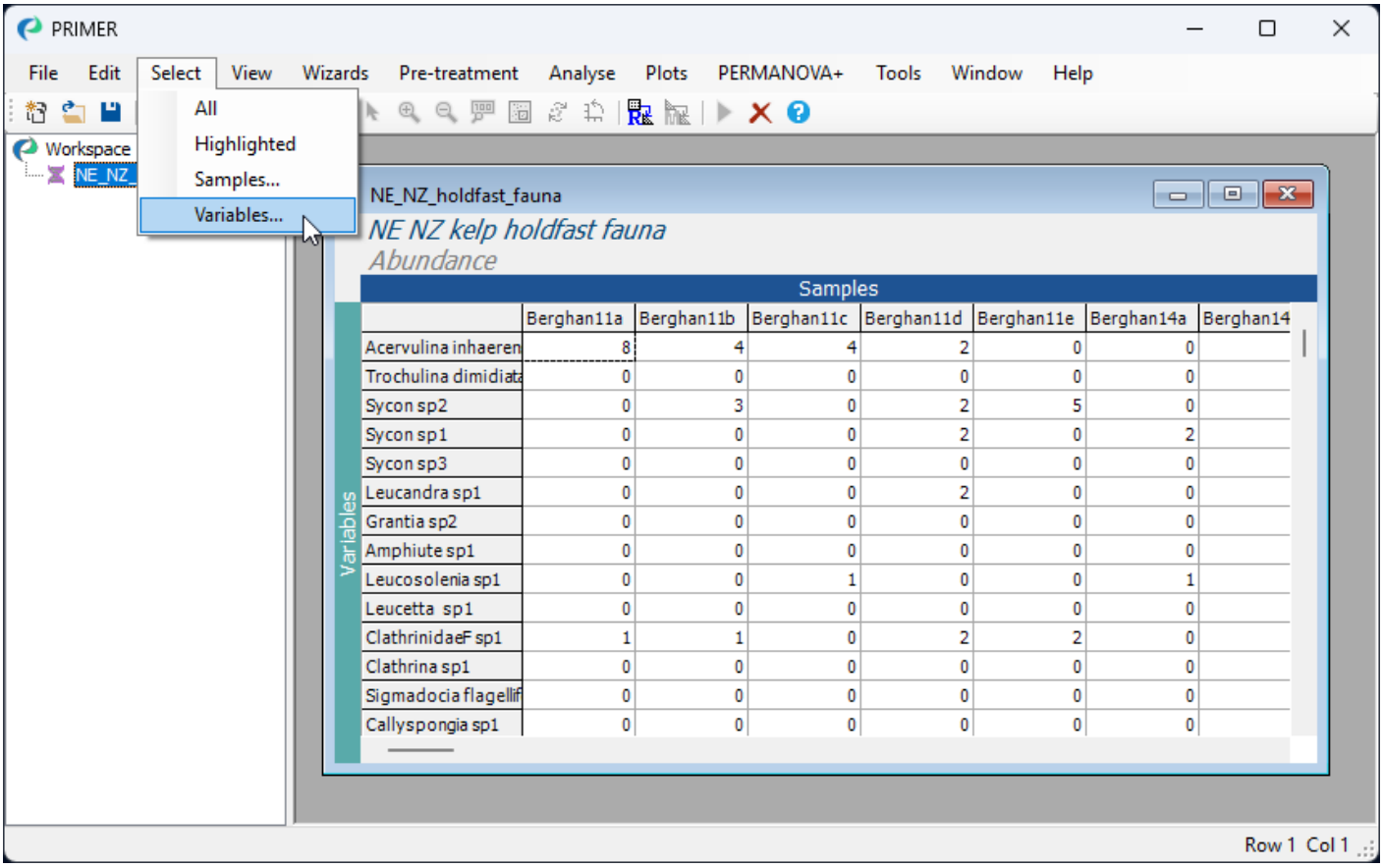
Label	Phylum	Class	Order	Family	Genus	Species	Ordinal
Acervulina inhaerens	Rhizopoda	Reticularea	Foraminiferida	Acervulinidae	Acervulina	Acervulina inha	N
Trochulina dimidiata	Rhizopoda	Reticularea	Foraminiferida	Discorbidae	Trochulina	Trochulina dimi	N
Sycon sp2	Porifera	Calcarea	Leucosolenida	Sycettidae	Sycon	Sycon sp2	N
Sycon sp1	Porifera	Calcarea	Leucosolenida	Sycettidae	Sycon	Sycon sp1	N
Sycon sp3	Porifera	Calcarea	Leucosolenida	Sycettidae	Sycon	Sycon sp3	N
Leucandra sp1	Porifera	Calcarea	Leucosolenida	Grantiidae	Leucandra	Leucandra sp1	N
Grantia sp2	Porifera	Calcarea	Leucosolenida	Grantiidae	Grantia	Grantia sp2	N
Amphiute sp1	Porifera	Calcarea	Leucosolenida	Grantiidae	Amphiute	Amphiute sp1	N
Leucosolenia sp1	Porifera	Calcarea	Leucosolenida	Leucosoleniidae	Leucosolenia	Leucosolenia sp	N
Leucetta sp1	Porifera	Calcarea	Clathrinida	Clathrinidae	Leucetta	Leucetta sp1	Y
ClathrinidaeF sp1	Porifera	Calcarea	Clathrinida	Clathrinidae	indet.	ClathrinidaeF sp	Y
Clathrina sp1	Porifera	Calcarea	Clathrinida	Clathrinidae	Clathrina	Clathrina sp1	Y
Sigmadocia flagellifer	Porifera	Demospongiae	Haplosclerida	Chalinidae	Sigmadocia	Sigmadocia fla	Y
Callyspongia sp1	Porifera	Demospongiae	Haplosclerida	Callyspongiidae	Callyspongia	Callyspongia sp	Y
Dysidea sp1	Porifera	Demospongiae	Dictyoceratida	Dysideidae	Dysidea	Dysidea sp1	Y
HalichondridaF sp1	Porifera	Demospongiae	Dictyoceratida	Halichondrida	indet.	HalichondridaF	Y
PhoriospongiidaeF1 s	Porifera	Demospongiae	Poecilosclerida	Phoriospongiida	indet.	Phoriospongiid	Y
PhoriospongiidaeF2 s	Porifera	Demospongiae	Poecilosclerida	Phoriospongiida	indet.	Phoriospongiid	Y
PhoriospongiidaeF3 s	Porifera	Demospongiae	Poecilosclerida	Phoriospongiida	indet.	Phoriospongiid	Y
Crella incrustans	Porifera	Demospongiae	Poecilosclerida	Crellidae	Crella	Crella incrustan	Y
Penares sp1	Porifera	Demospongiae	Astrophorida	Ancorinidae	Penares	Penares sp1	Y
DemospongiaeC sp1	Porifera	Demospongiae	indet.	indet.	indet.	DemospongiaeC	Y
Sertularella simplex	Cnidaria	Hydrozoa	Conica (subCla	Sertulariidae	Sertularella	Sertularella sim	Y
Sertularia sp	Cnidaria	Hydrozoa	Conica (subCla	Sertulariidae	Sertularia	Sertularia sp	Y
Plumularia setacea	Cnidaria	Hydrozoa	Conica (subCla	Plumulariidae	Plumularia	Plumularia seta	Y

Click **OK** on the 'Indicators' dialog, so the data matrix is the active item in the workspace again.

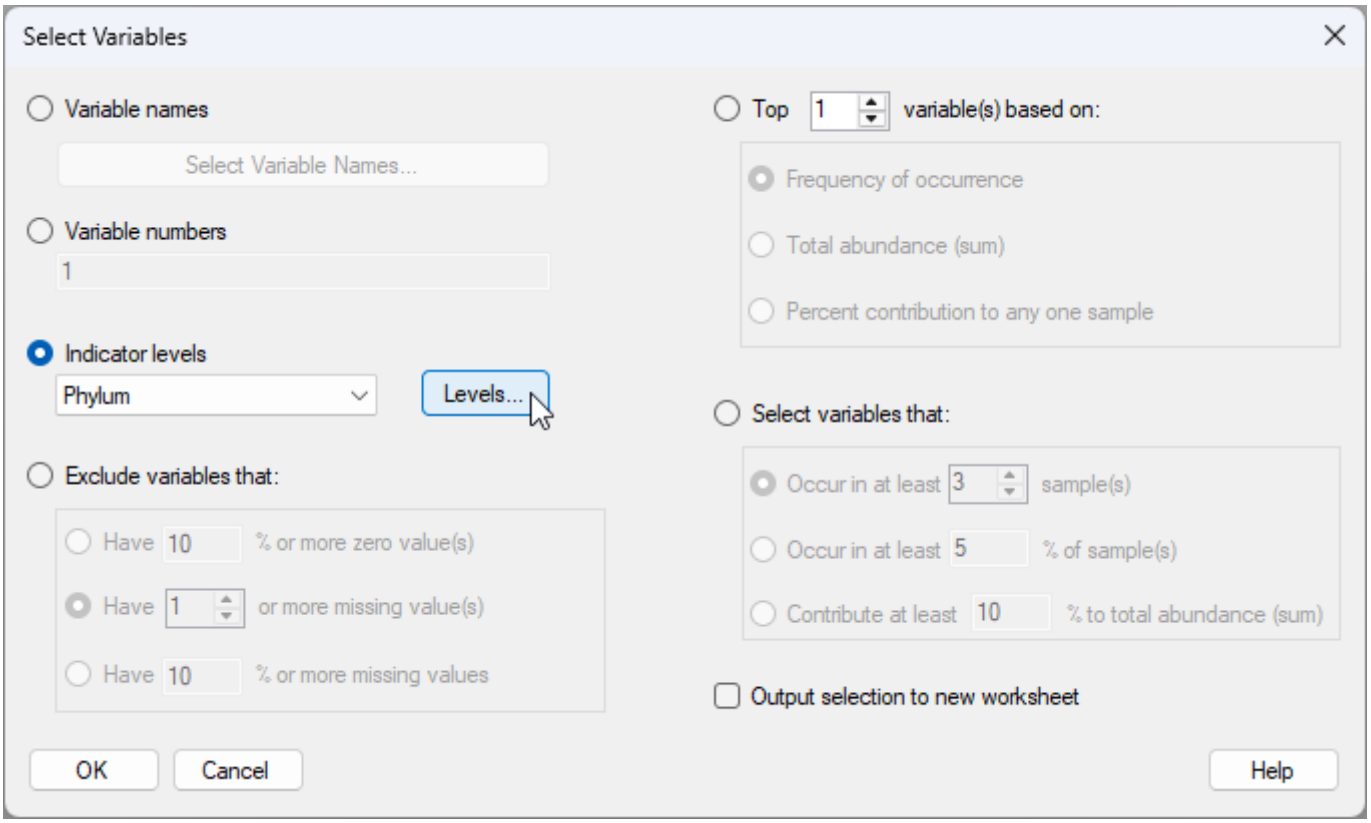
Select a subset of variables, using an indicator

We wish to select just the mollusc species for the analysis.

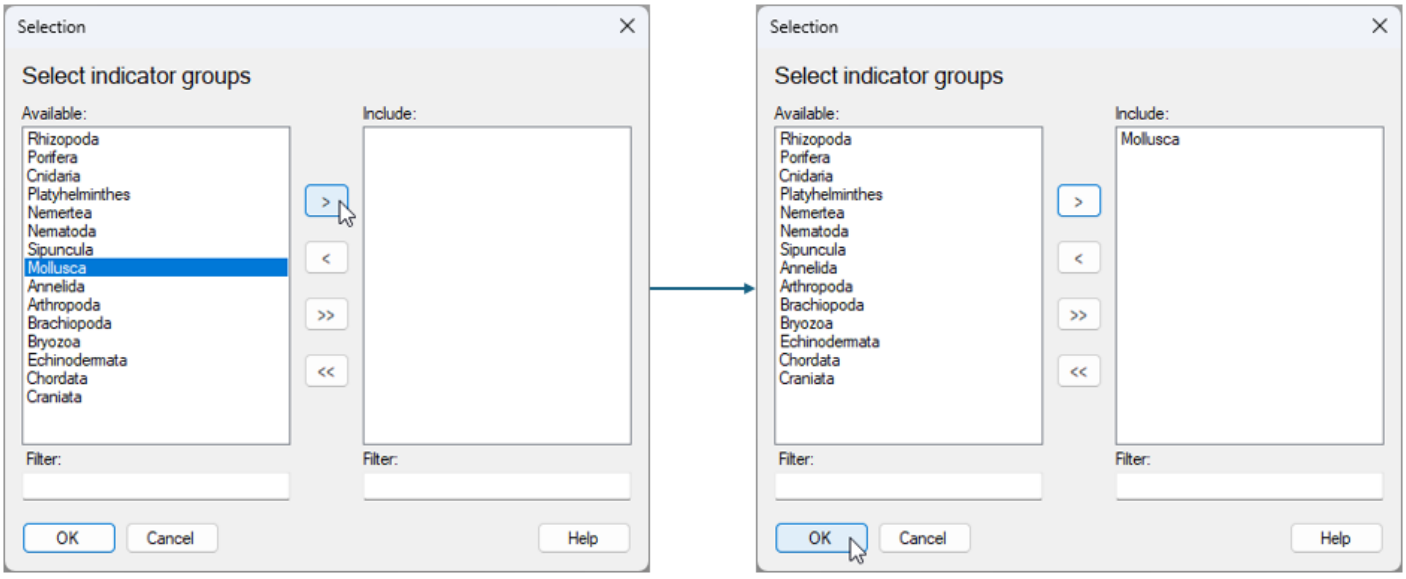
1. From the 'NE_NZ_holdfast_fauna.pri' data sheet, click **Select** > **Variables...**



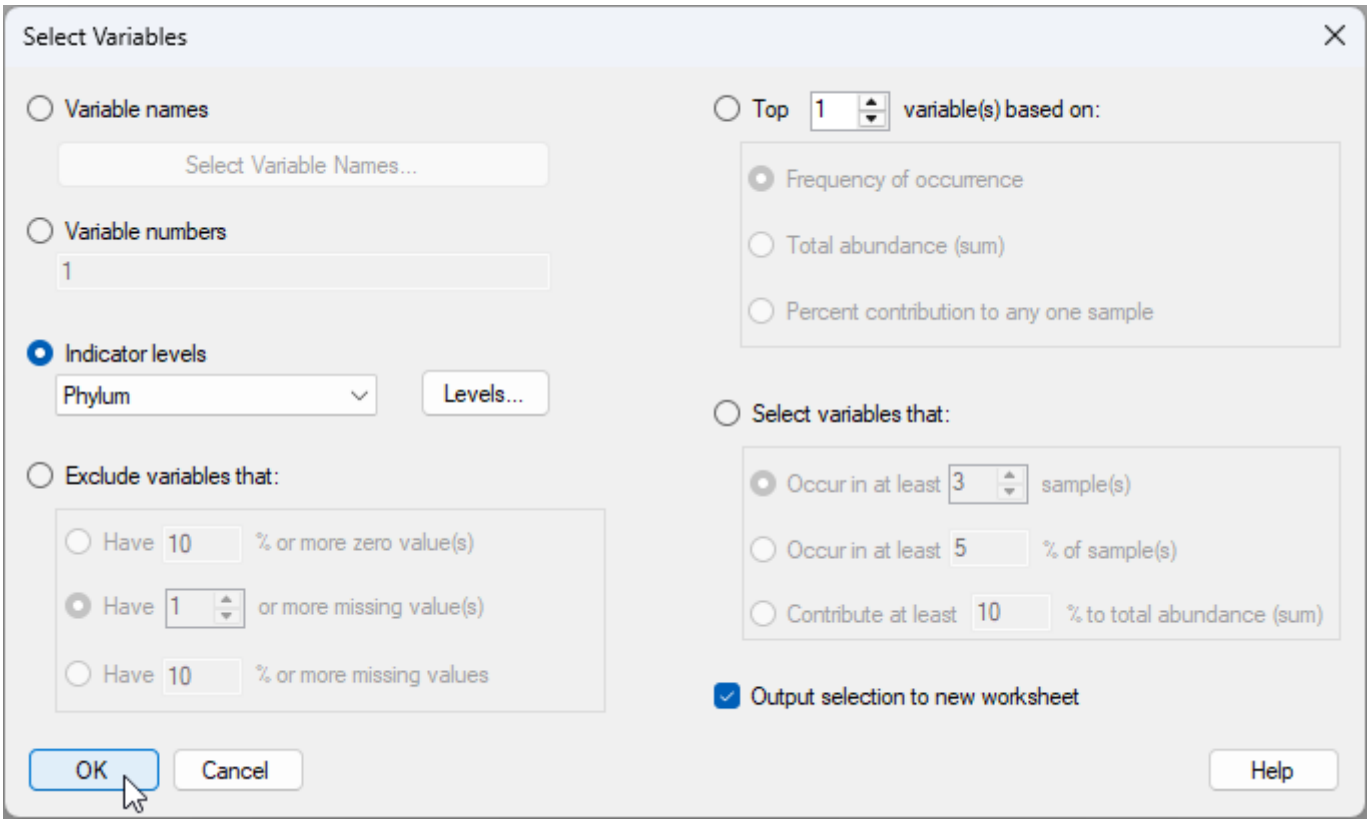
2. In the 'Select Variables' dialog, choose ($\bullet$Indicator levels > Phylum) and click **Levels...**.



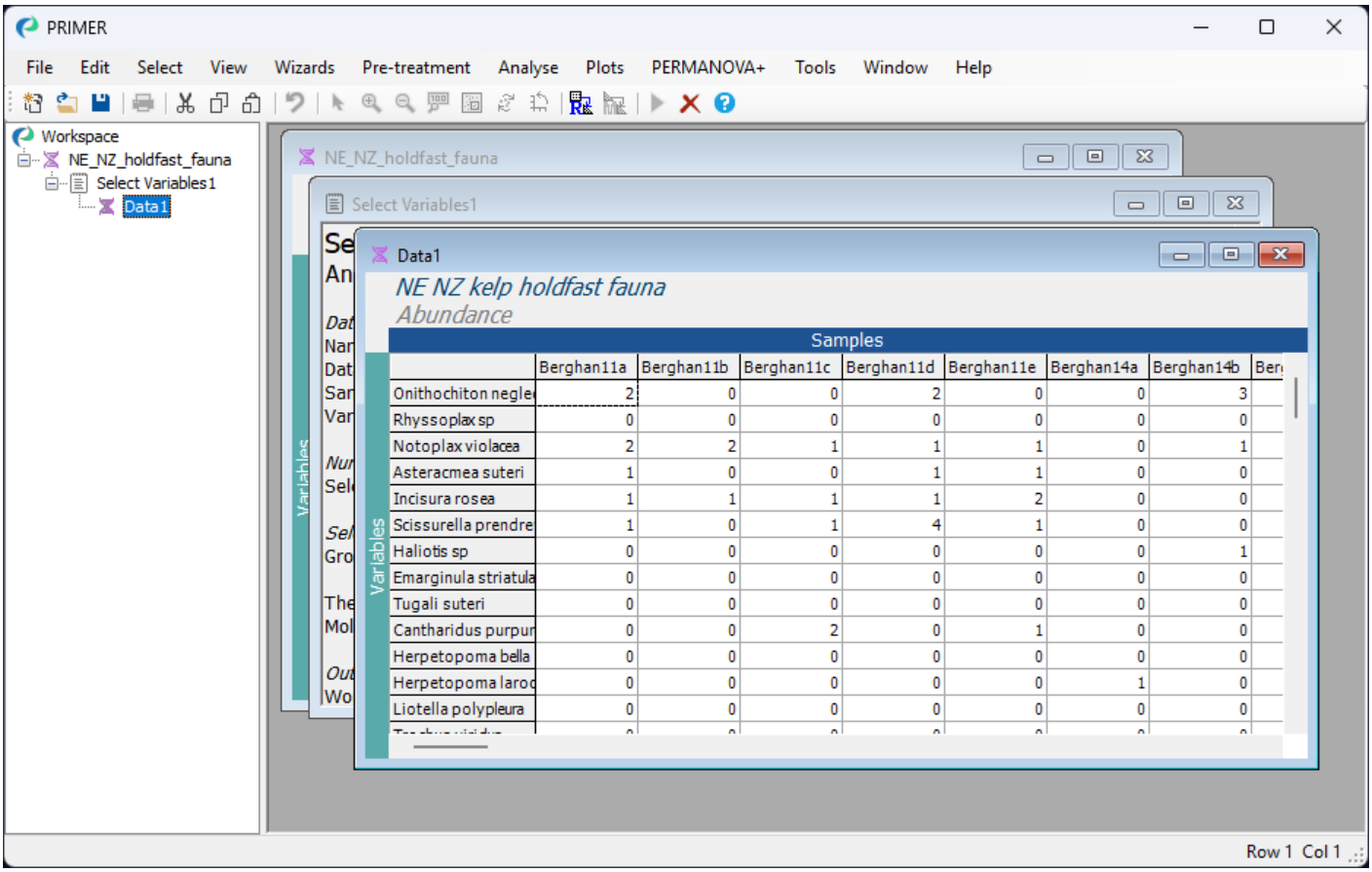
3. In the 'Selection' dialog, click on the word 'Mollusca' in the list of 'Available' phylum categories, and click on the single-right-arrow button:  . This will move the word 'Mollusca' over to the 'Include:' box on the right-hand side of the 'Selection' dialog window. Click **OK**.



4. Now that we have selected the Molluscs, and we are back in the 'Select Variables' dialog window, we *also* want to choose to '$\checkmark$ Output selection to new worksheet', as shown below. This will kick out just the variables that we have selected into a new worksheet for ensuing analysis. We can now click **OK** on the 'Select Variables' dialog.



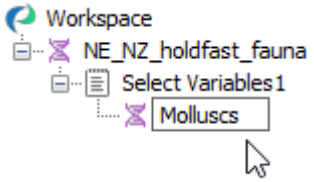
Voila! Now you have just the mollusc species alone in a new worksheet called 'Data1'¶.



Rename the selected subset of data

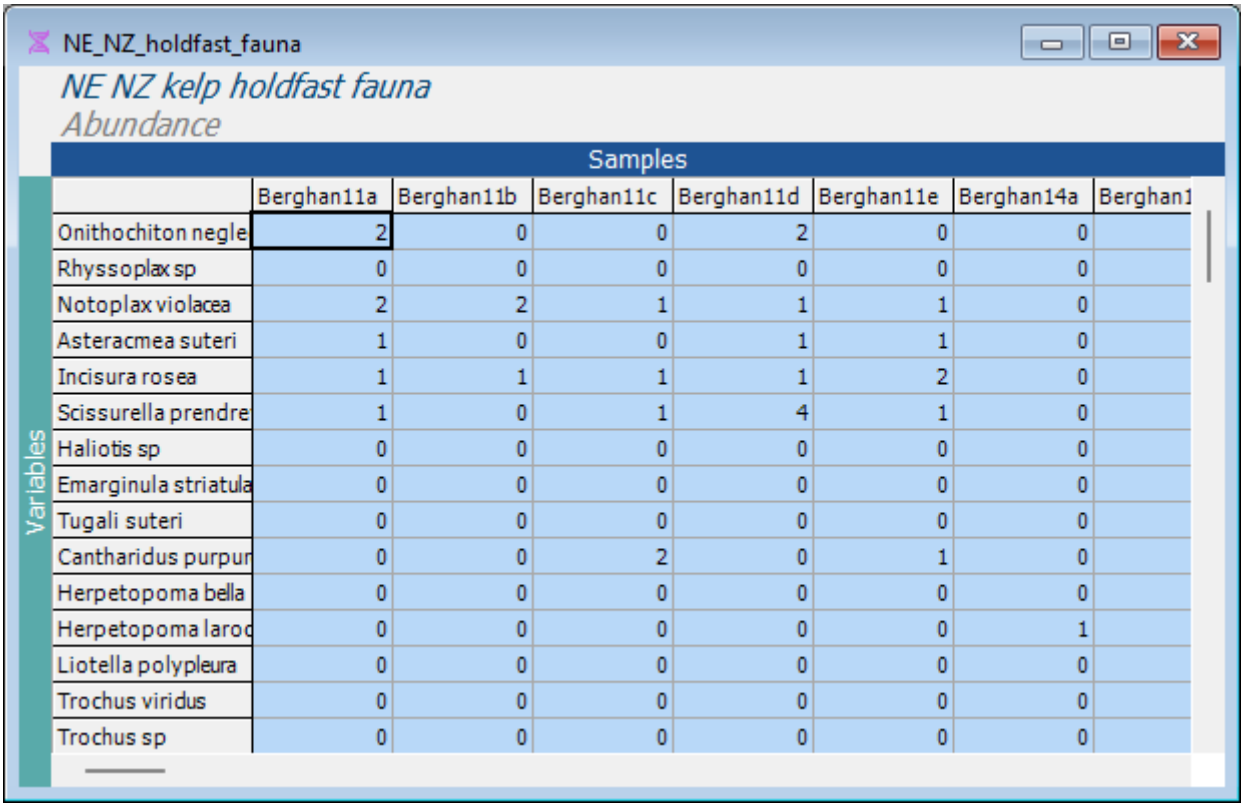
From the subsetted data matrix ('Data1'), if you click on **Edit** > **Properties...**, you will see that this new sheet now only has 105 variables ('Number of rows:').

To change the name of the data sheet, click, hover and click again on the name 'Data1' in the Explorer tree window (*or* click **File** > **Rename Data** *or* hit the 'F2' key) and type in a new name for the subsetted data sheet: Molluscs.



At this point, you might like to save your workspace. Click **File** > **Save Workspace As...** > (Filename: NZ_holdfast_molluscs.pwk).

¶ *An aside: Note that whenever you have selected a subset of data (this might be a subset of variables, as done here, or a subset of samples, or both), then the original data matrix on which the operation has been done will have a blue background colour to indicate that you have done this, like so:*

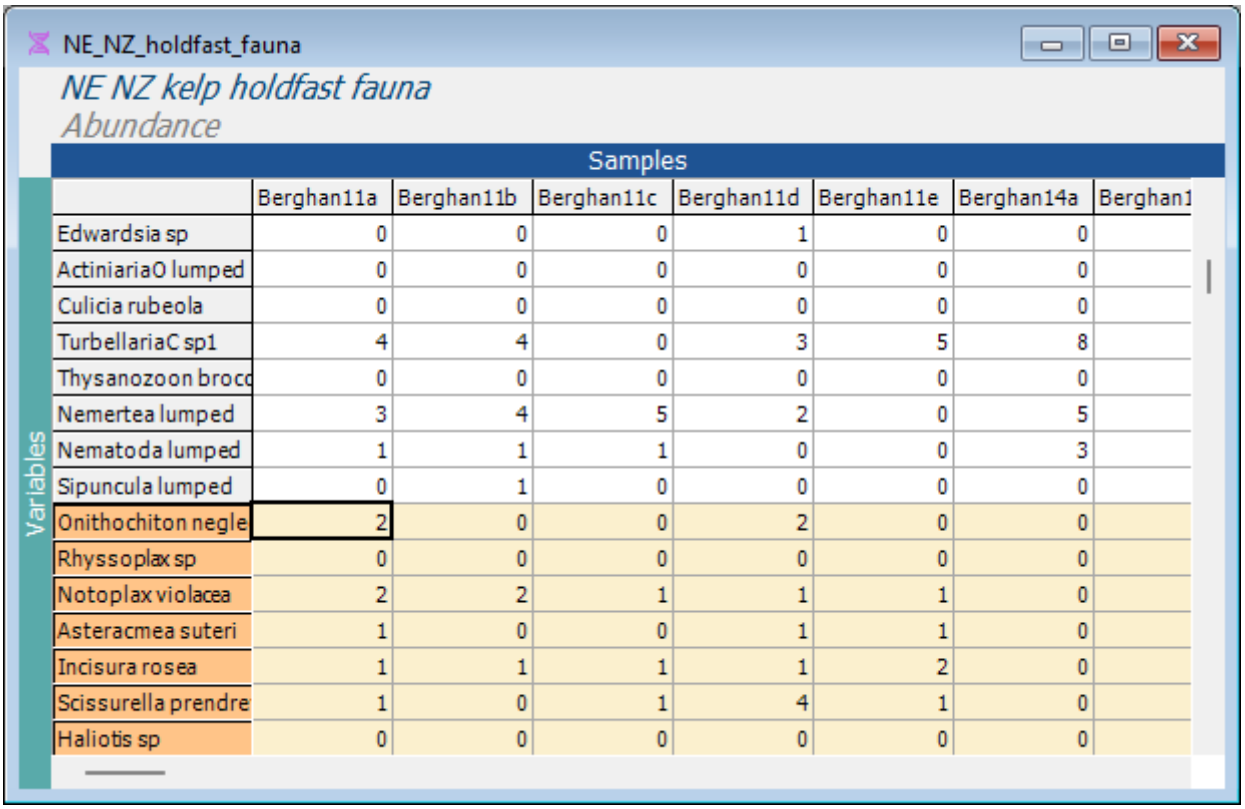


	Berghan11a	Berghan11b	Berghan11c	Berghan11d	Berghan11e	Berghan14a	Berghan1
Onithochiton negle	2	0	0	2	0	0	
Rhyssoplax sp	0	0	0	0	0	0	
Notoplax violacea	2	2	1	1	1	0	
Asteracmea suteri	1	0	0	1	1	0	
Incisura rosea	1	1	1	1	2	0	
Scissurella prendre	1	0	1	4	1	0	
Haliotis sp	0	0	0	0	0	0	
Emarginula striatula	0	0	0	0	0	0	
Tugali suteri	0	0	0	0	0	0	
Cantharidus purpur	0	0	2	0	1	0	
Herpetopoma bella	0	0	0	0	0	0	
Herpetopoma laroc	0	0	0	0	0	1	
Liotella polypleura	0	0	0	0	0	0	
Trochus viridus	0	0	0	0	0	0	
Trochus sp	0	0	0	0	0	0	

Any analyses done on a selected subset of data will only be performed on that subset. It is usually a good idea to duplicate and rename a selected subset of data, so as to keep any analysis done on that subset of data clear and separate from the (full) original dataset. To duplicate an item in the Explorer tree, click Tools > Duplicate. To rename the duplicated item, hit F2 (or click File > Rename Data).

Note that subsetting does not affect the original full data matrix of information in any way, which is still always there. You can clear any subset selection (of variables and/or samples) by clicking on

Select > All to return to the full data matrix in its entirety. The blue background colour will go away when you do that, and the formerly selected data will yet be highlighted in yellow-orange hues.



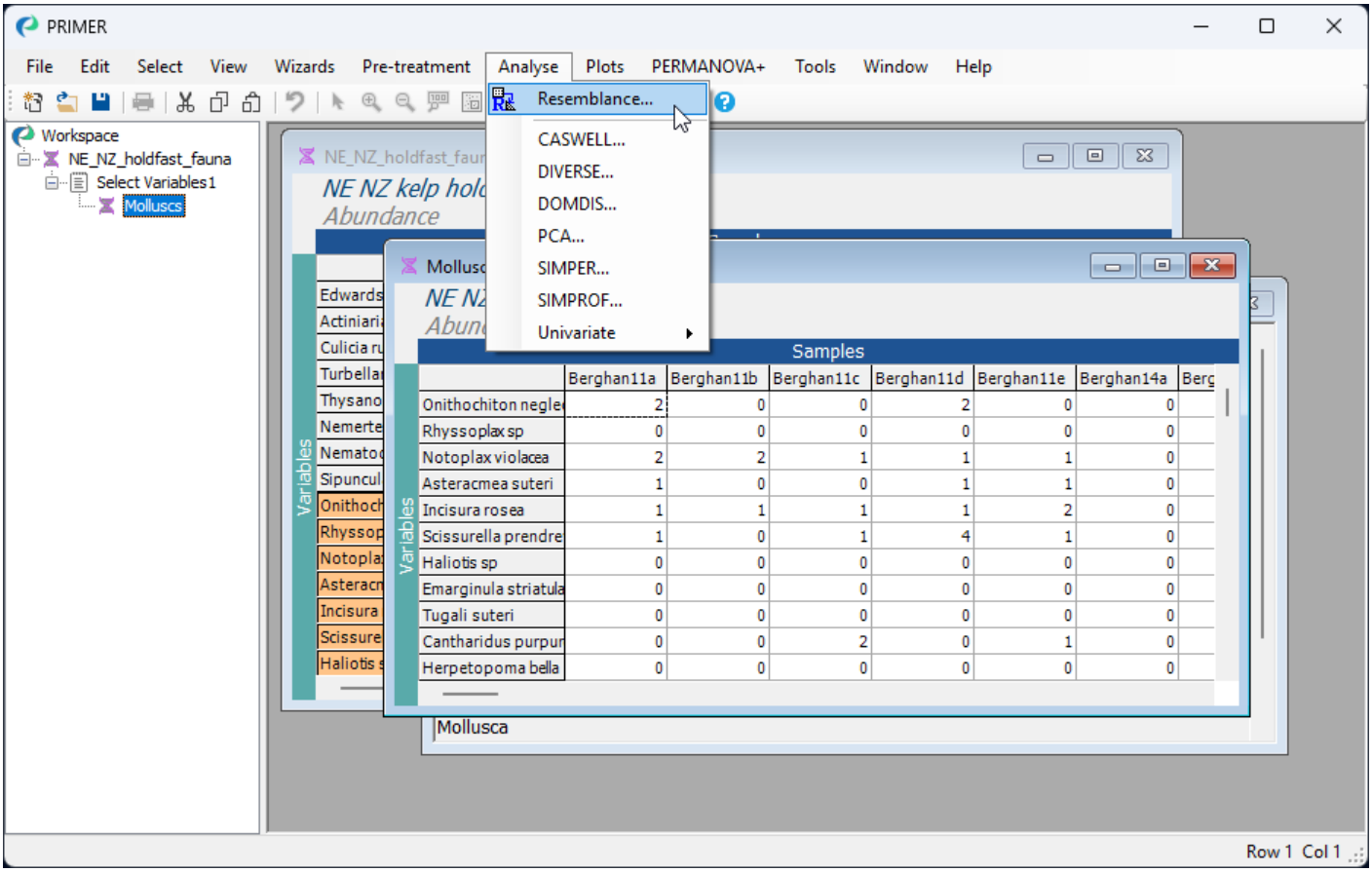
	Berghan11a	Berghan11b	Berghan11c	Berghan11d	Berghan11e	Berghan14a	Berghan1
Edwardsia sp	0	0	0	1	0	0	
ActiniariaO lumped	0	0	0	0	0	0	
Culicia rubeola	0	0	0	0	0	0	
TurbellariaC sp1	4	4	0	3	5	8	
Thysanozoon broc	0	0	0	0	0	0	
Nemertea lumped	3	4	5	2	0	5	
Nematoda lumped	1	1	1	0	0	3	
Sipuncula lumped	0	1	0	0	0	0	
Onithochiton negle	2	0	0	2	0	0	
Rhyssoplax sp	0	0	0	0	0	0	
Notoplax violacea	2	2	1	1	1	0	
Asteracmea suteri	1	0	0	1	1	0	
Incisura rosea	1	1	1	1	2	0	
Scissurella prendre	1	0	1	4	1	0	
Haliotis sp	0	0	0	0	0	0	

Clicking on Select > Highlighted can then be used to re-instate the selection from before, if desired.

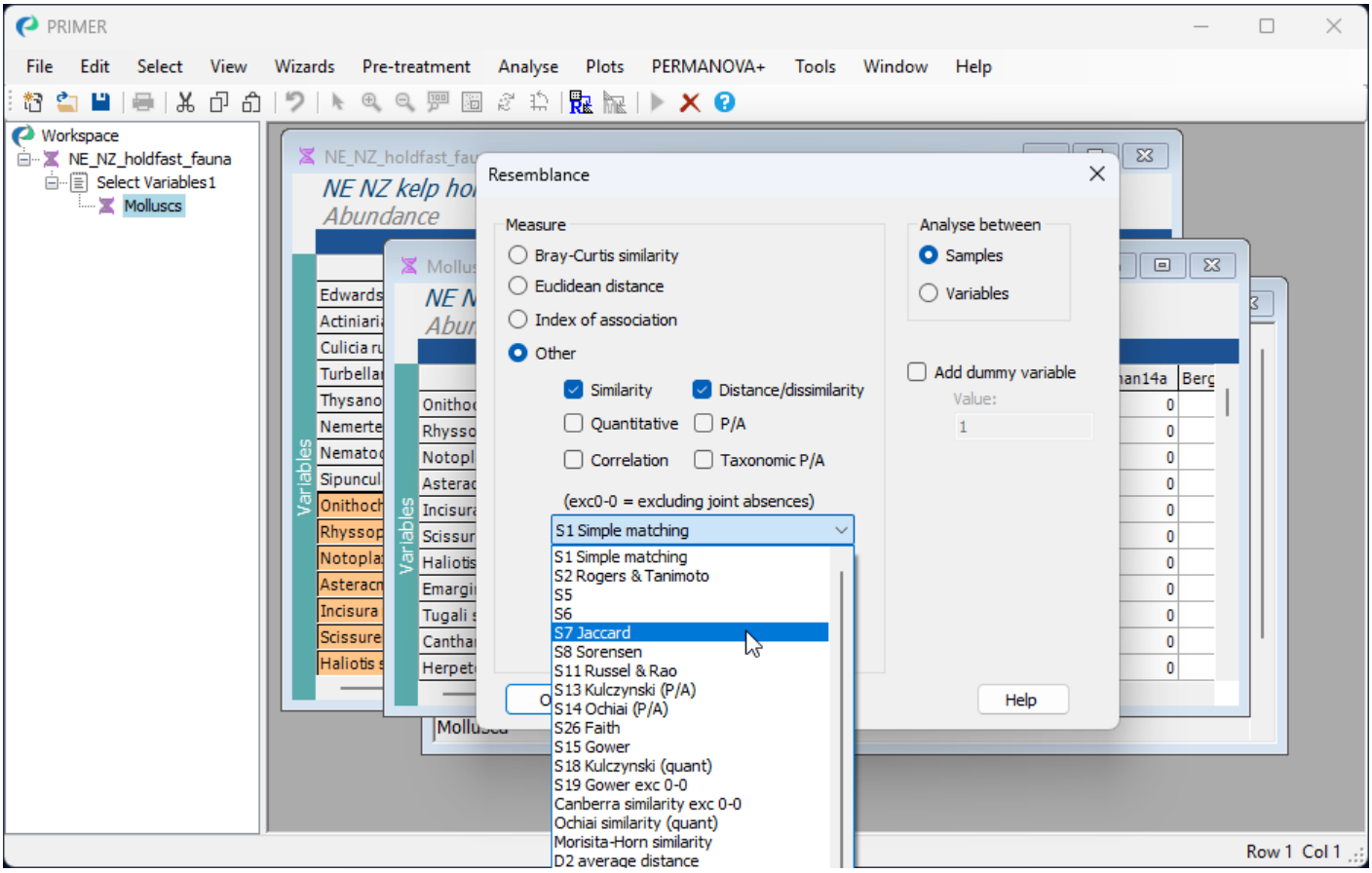
Step 2: Jaccard resemblance

Calculate the Jaccard resemblance

From the 'Molluscs' data sheet, click **Analyse** > **Resemblance...**.

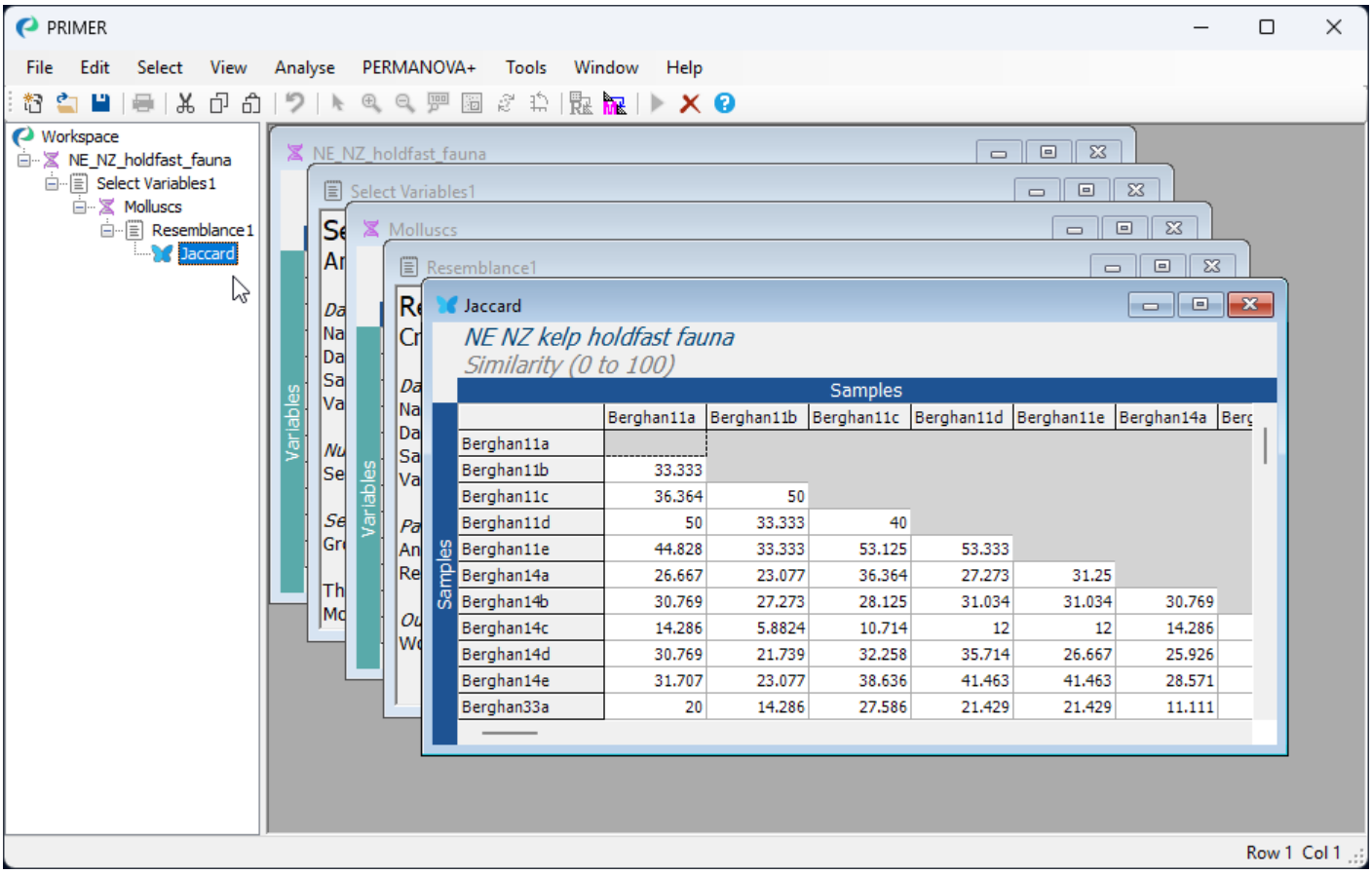


In the 'Resemblance' dialog, choose ($\bullet$Other) and then click on the drop-down menu to find 'S7 Jaccard', then click **OK**. (Note: 'S7' refers to the nomenclature used by Legendre & Legendre (2012) in their Chapter 7 on measures of ecological resemblance).



This produces a Jaccard similarity matrix among all pairs of holdfasts, called 'Resem1' by default. A nice feature of the Jaccard similarity measure is that it is directly interpretable as the percentage of shared species. For example, the first two holdfasts in the data matrix have a Jaccard similarity of 33.33%, so approximately one-third of the species that occur in either one or the other holdfast occur in both of them (i.e., are jointly present).

You can re-name this 'Jaccard' (click on the name 'Resem1' in the Explorer tree and hit the 'F2' key to re-name it), for clarity in what follows.



Samples	Berghan11a	Berghan11b	Berghan11c	Berghan11d	Berghan11e	Berghan14a	Berg
Berghan11a							
Berghan11b	33.333						
Berghan11c	36.364	50					
Berghan11d	50	33.333	40				
Berghan11e	44.828	33.333	53.125	53.333			
Berghan14a	26.667	23.077	36.364	27.273	31.25		
Berghan14b	30.769	27.273	28.125	31.034	31.034	30.769	
Berghan14c	14.286	5.8824	10.714	12	12	14.286	
Berghan14d	30.769	21.739	32.258	35.714	26.667	25.926	
Berghan14e	31.707	23.077	38.636	41.463	41.463	28.571	
Berghan33a	20	14.286	27.586	21.429	21.429	11.111	

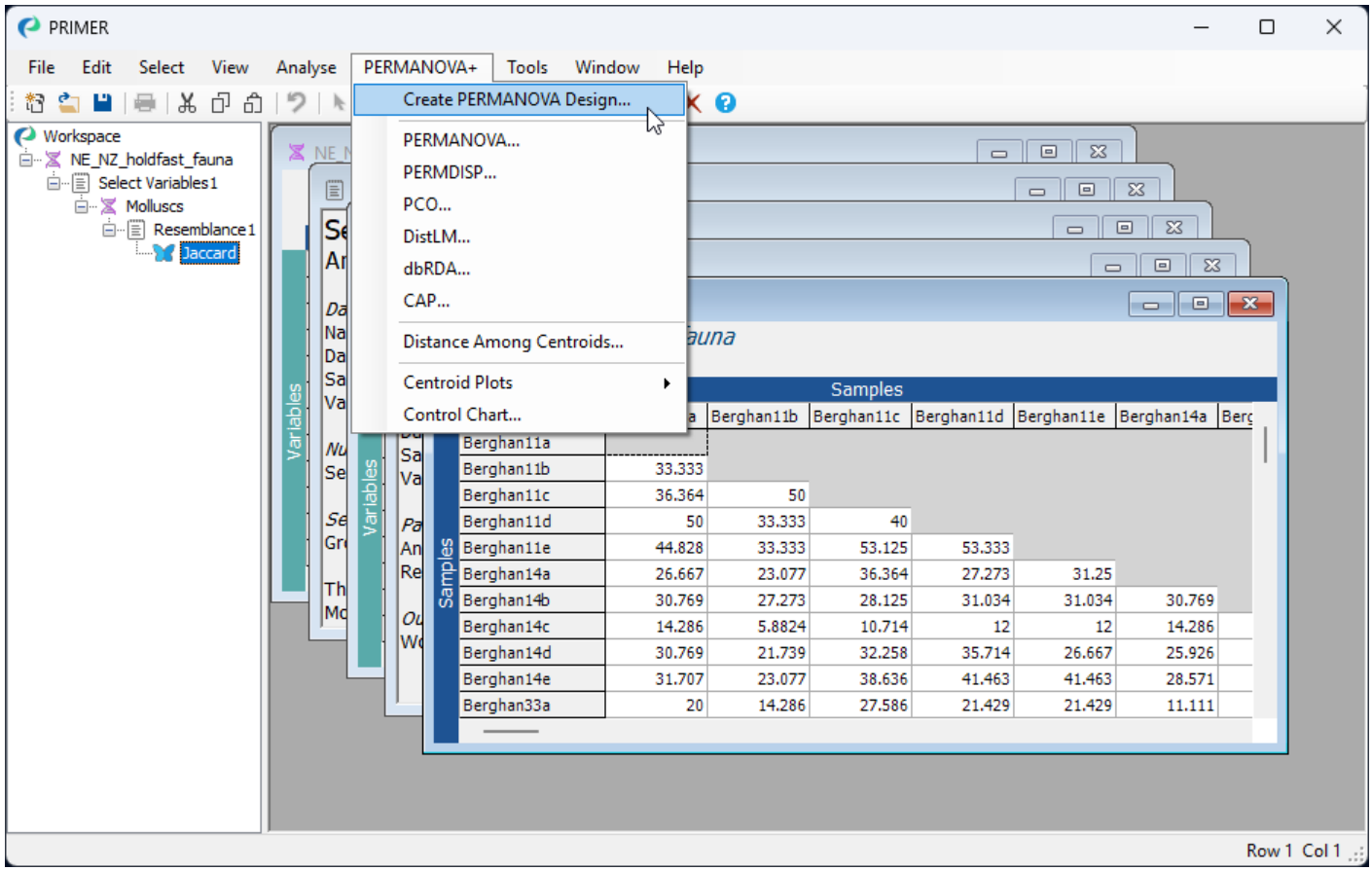
Step 3: Specify the design

PERMANOVA requires a **design file** to run.

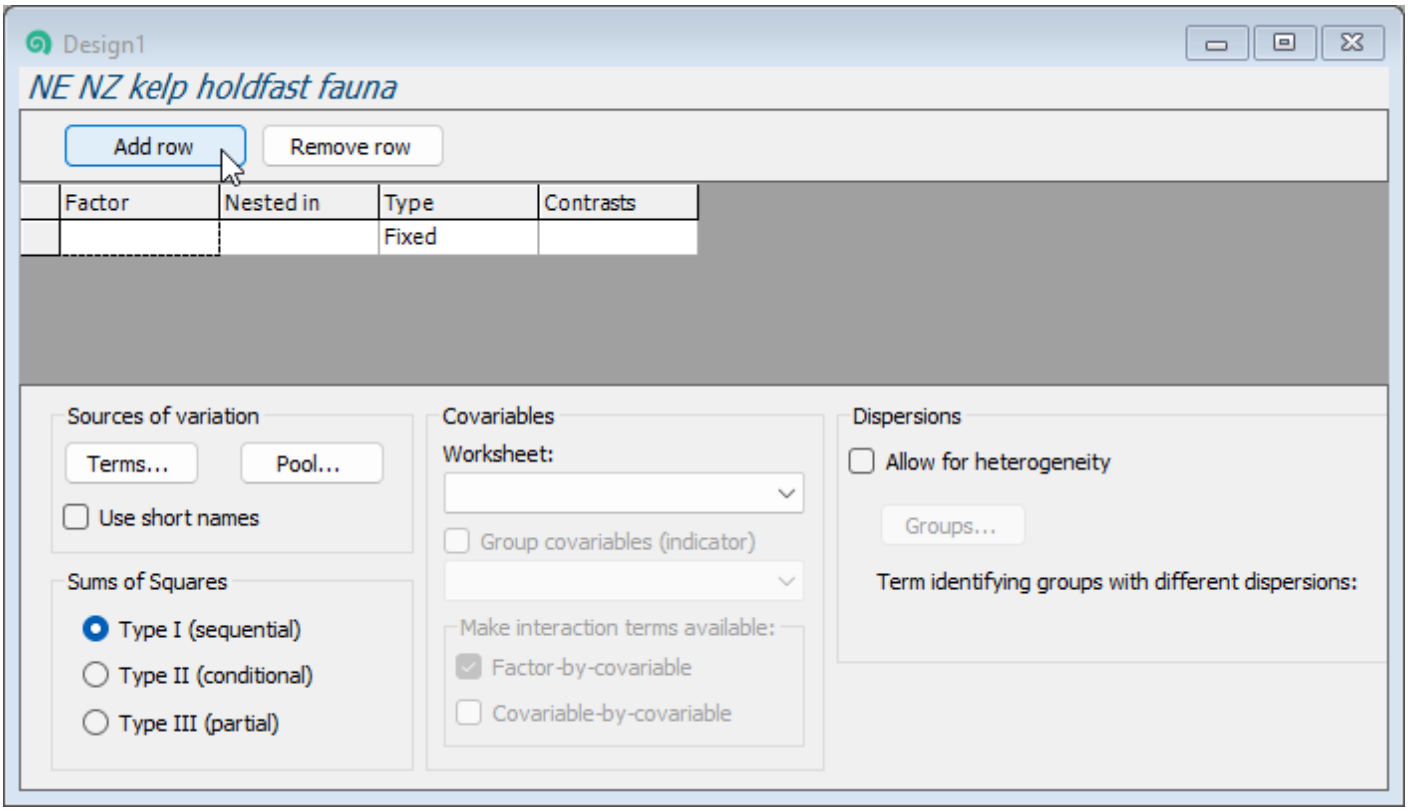
You can see the Factors associated with the holdfast data matrix (or its resemblance matrix) by clicking on **Edit** > **Factors...**. These factors will be 'visible' to the PERMANOVA dialog that we will use to create our design file.

For this study, we want to create a design file that has all of the information that PERMANOVA will need to construct the correct partitioning, the correct pseudo-F ratios and the correct permutation algorithms to test every term in the model that is implied by the design. For this example, we have a fully hierarchical (nested) study design with three random factors: Locations, Sites (within Locations) and Areas (within Sites).

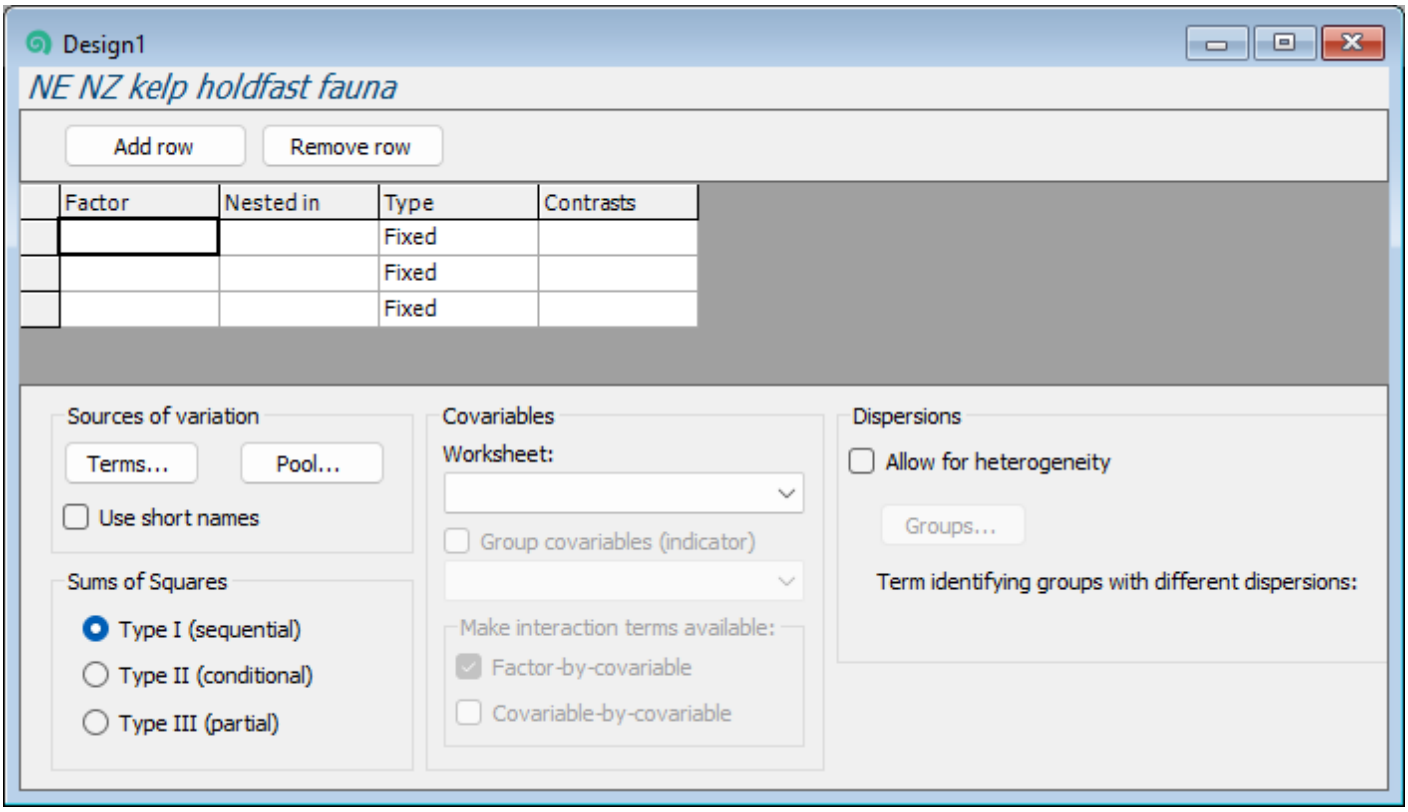
1. From the resemblance matrix ('Jaccard'), click **PERMANOVA+** > **Create PERMANOVA Design...**.



2. You will see a design file, which always is indicated in the Explorer tree by the symbol , which you can use to specify all pertinent aspects of the sampling / experimental design‡. Initially, it will only be shown with a single row. There need to be as many rows as there are factors in your design. So, here, we will add two more rows by clicking on the 'Add row' button twice:



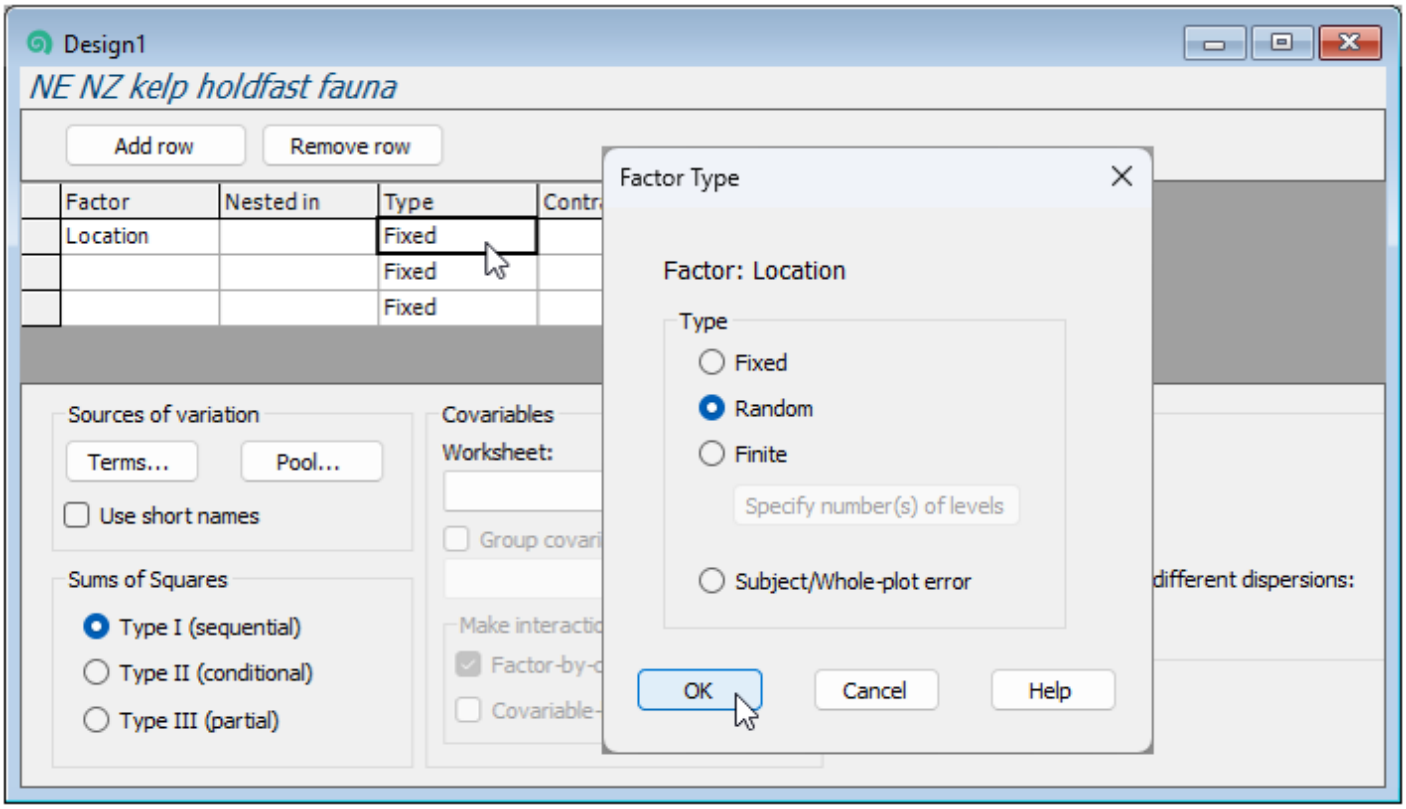
This yields:



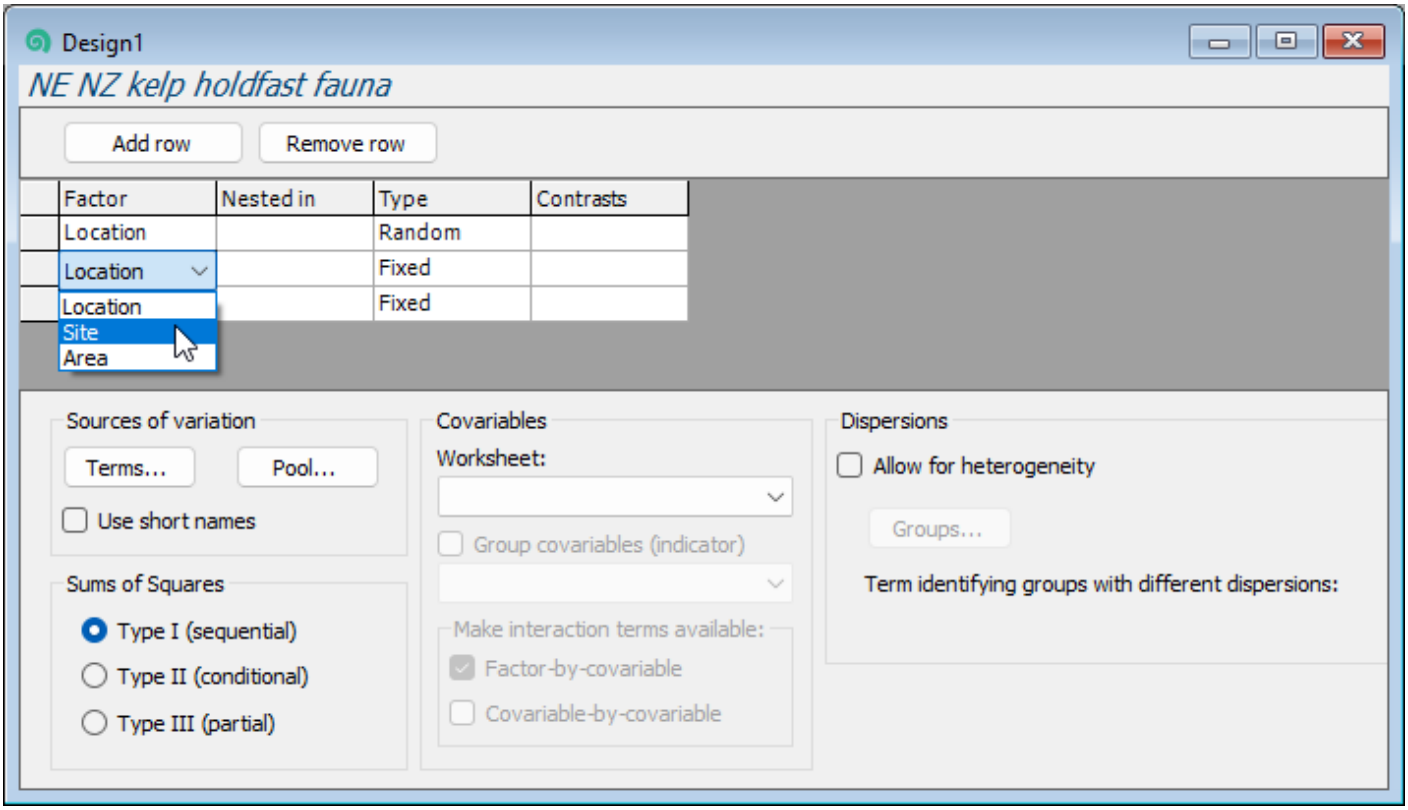
Next, you will need to specify, in turn, the name and properties of each factor for the analysis in its own row.

4. **Location:** First, in row 1, double-click in the blank cell under the word 'Factor', and you will see a drop-down menu listing all of the factors associated with the resemblance matrix from which this design file was created. Choose 'Location' to fill this cell. Location

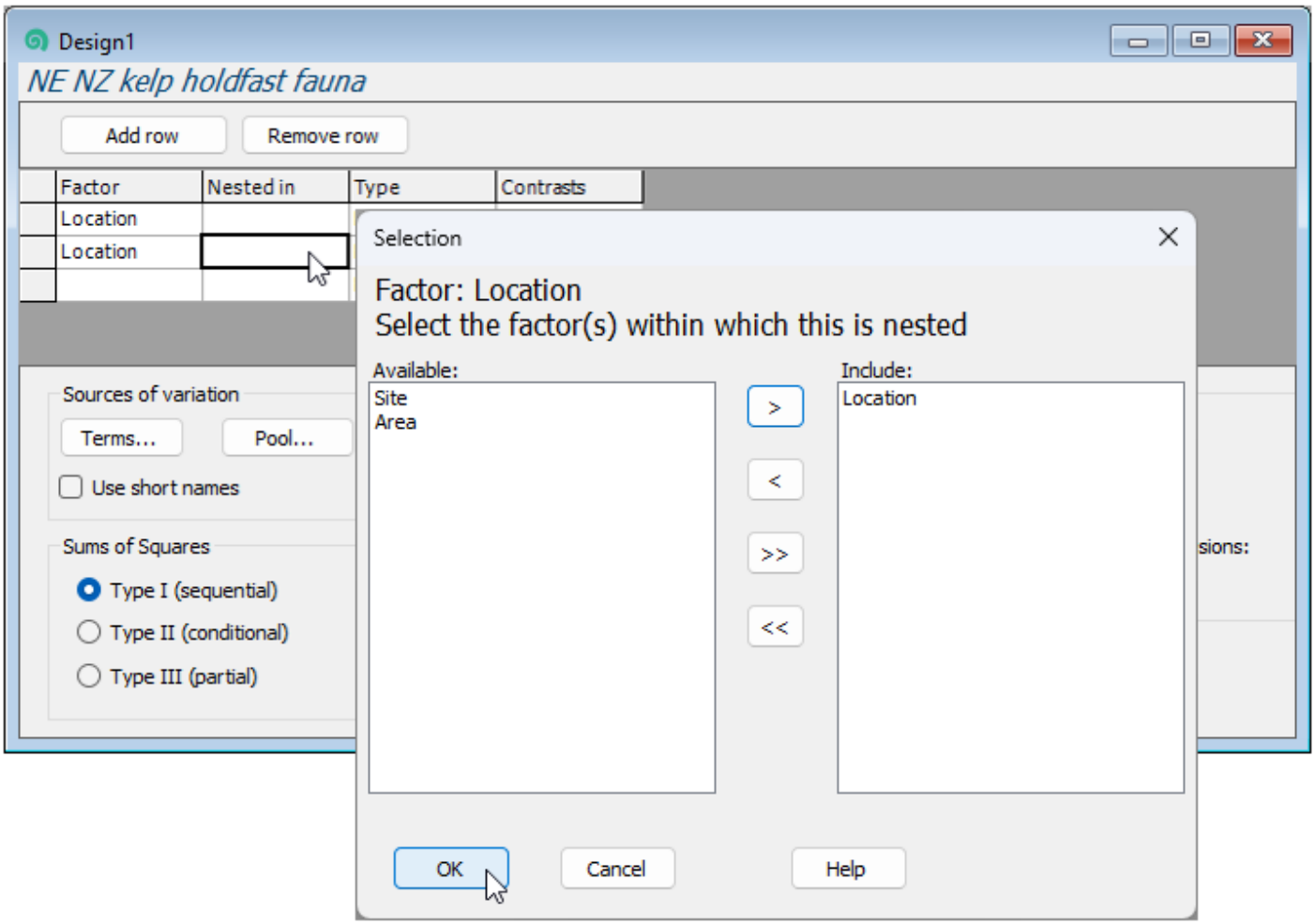
is not nested in anything, so we leave the second cell in row 1 blank. In the third cell of row 1, we have to specify that Location is a **random** factor, so double-click on the word 'Fixed' and in the 'Factor Type' dialog window under 'Type', click on $\bullet$Random, then click **OK**.



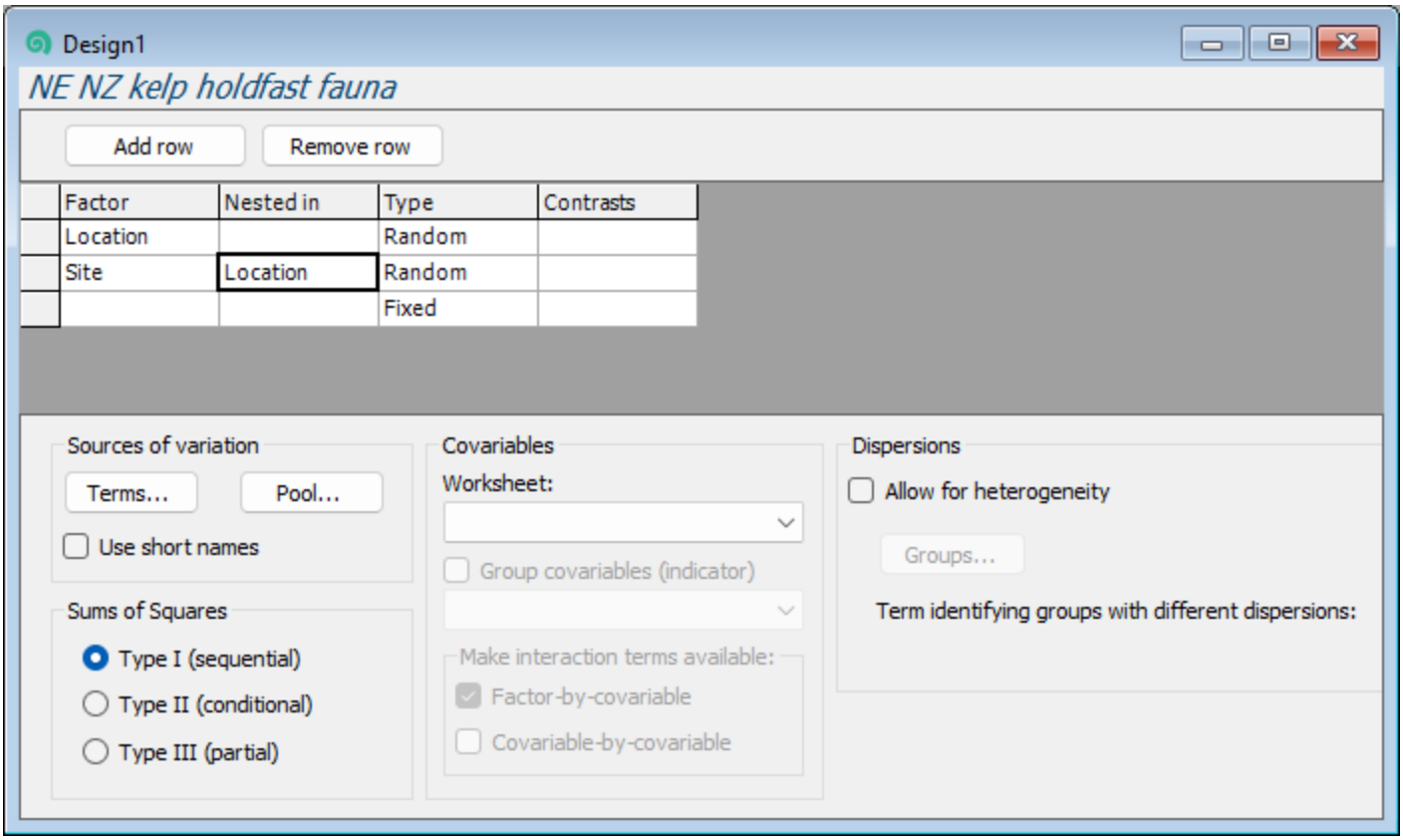
5. **Site:** Next, we need to specify the second factor in the design in row 2 of the design file. Double-click on the cell in row 2 of the 'Factor' column (column 1) and choose 'Site'.



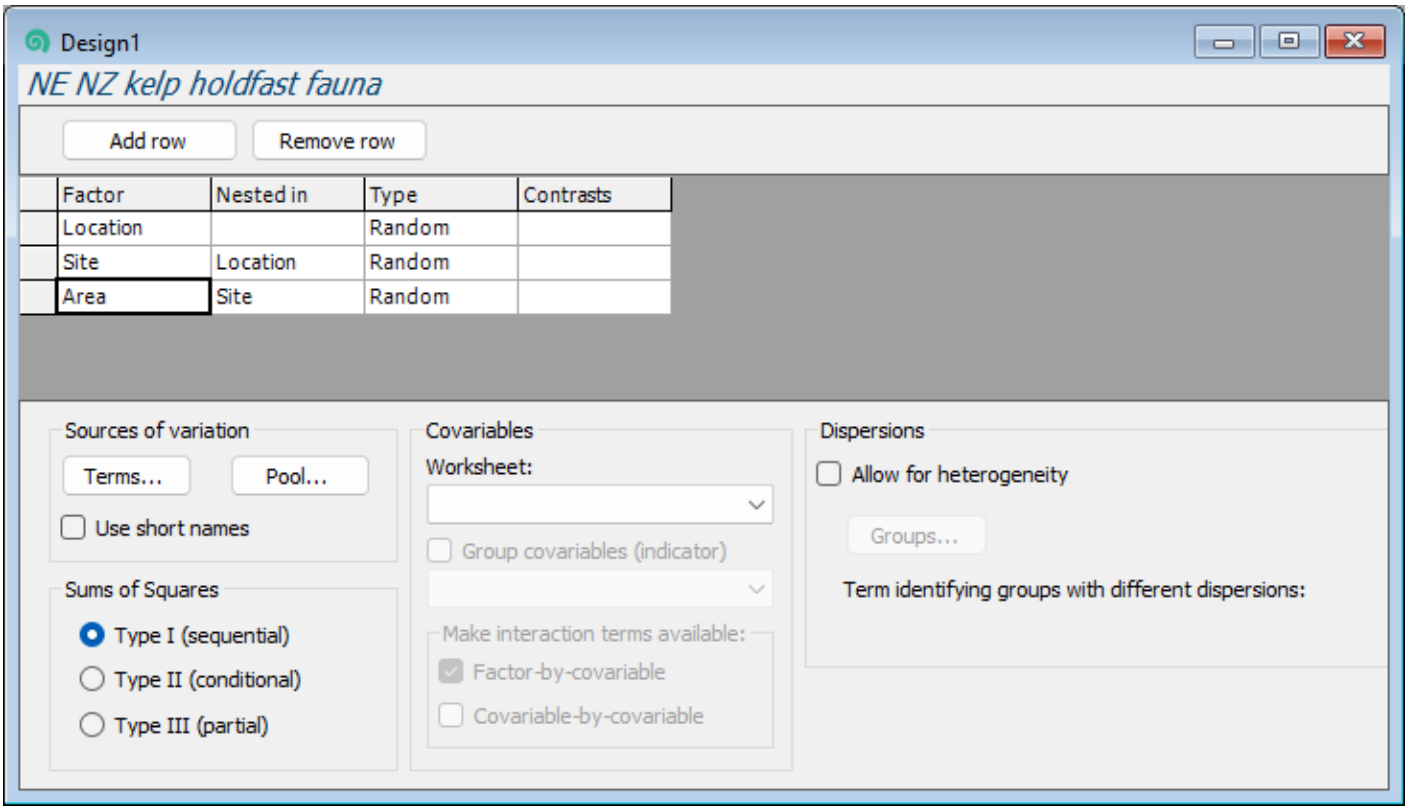
6. Sites are **nested in Locations**, so we have to specify that in column two ('Nested in'), accordingly. Double-click on the cell in row 2 in column 2 and a 'Selection' dialog will pop up to allow you to choose the factors within which 'Site' is nested. You will need to click on the word 'Location' (in the 'Available:' box on the left), then on the single-right-arrow button (>) to move it over into the 'Include:' box (on the right), then click **OK**, like so:



7. Make sure that the factor 'Site' is also specified in column 3 as 'Random'. (This happens automatically after specifying a nested term in column 2, because nested terms are, almost always, random factors.) Your design file should now look like this:

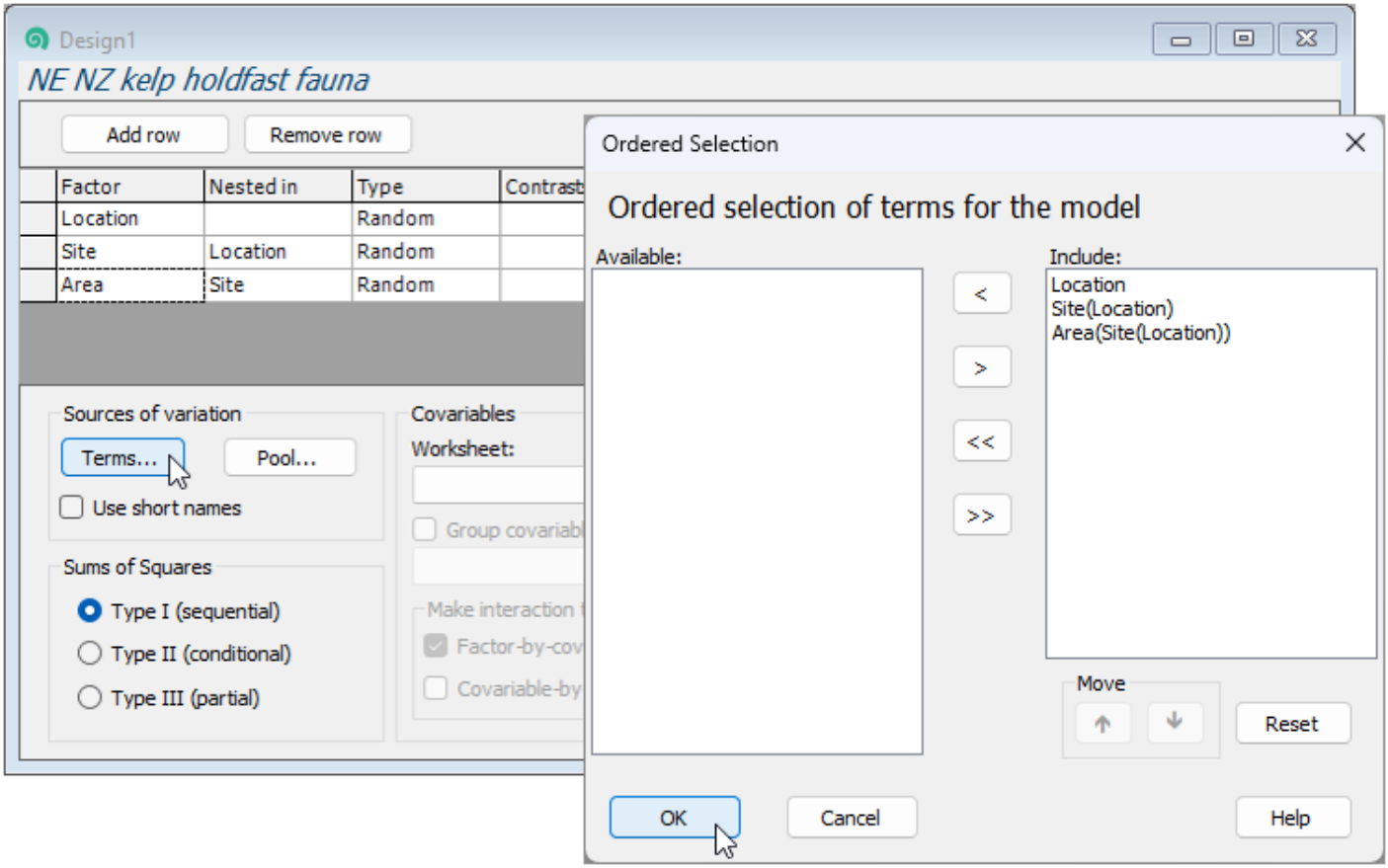


8. **Area:** Finally, we need to specify the third factor (Areas) correctly in row 3 of the design file. Under 'Factor' choose 'Area'. Under 'Nested in', specify 'Site', and make sure that column three has the word 'Random'. The final three-way nested design file should look like this:



Now our design file has all of the relevant information to run the PERMANOVA analysis.

You can see all of the terms in your PERMANOVA model by clicking on the 'Terms' button, as shown below:



(Nb. There are no interaction terms in this particular fully nested hierarchical design, but if interactions are implied by your design e.g., in crossed designs, then all such interaction terms, by default, will be included in the model.)

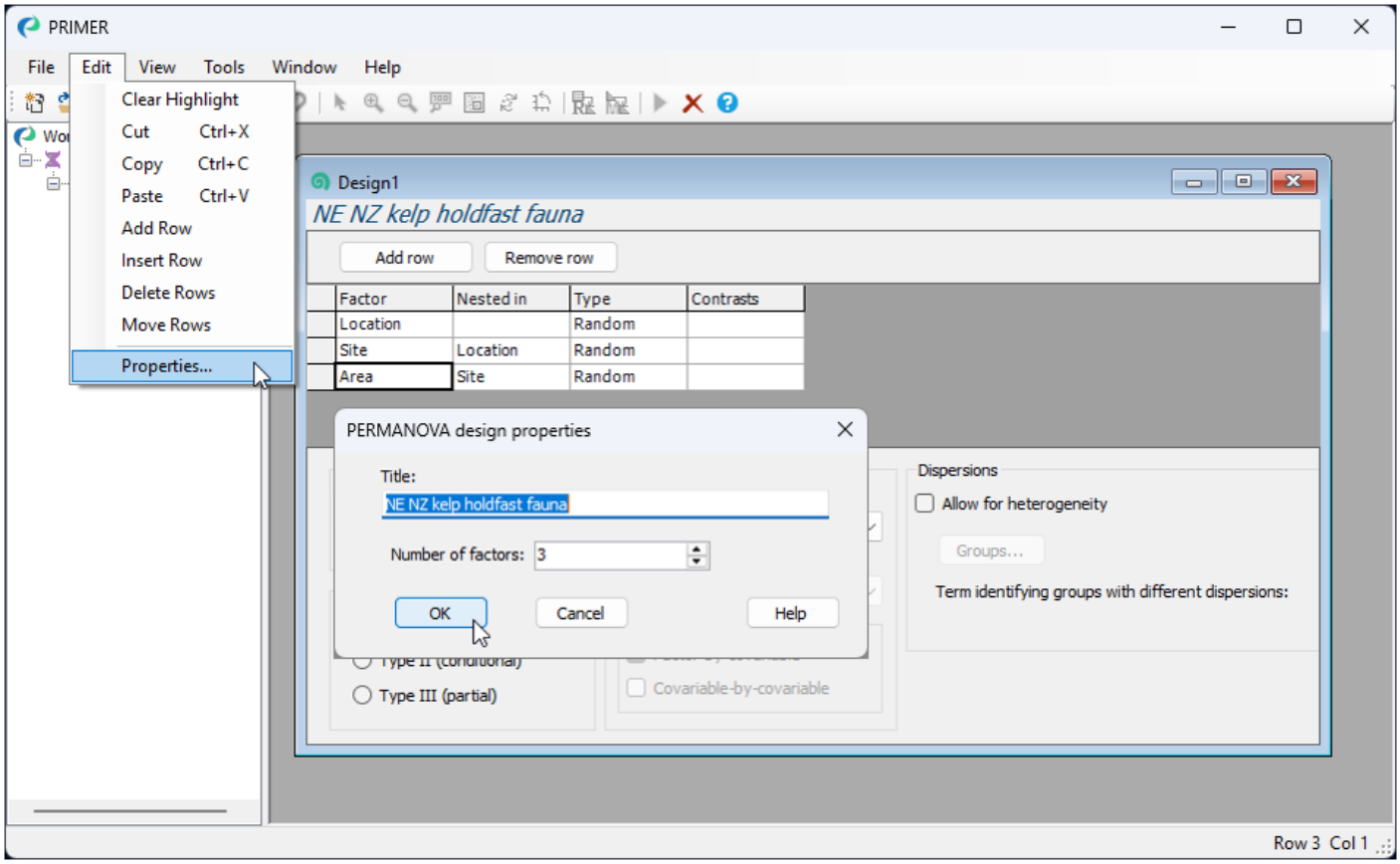
You will note that there are other options here, including the ability to:

- change the model by removing or pooling terms, or change their order,
- include tests of specific contrasts,
- choose the 'Type' of sums of squares (e.g. for unbalanced cases),
- add quantitative covariates; and/or
- accommodate heterogeneity of dispersions, etc.

Here, we shall leave all of these as their defaults. We are now ready to run the PERMANOVA analysis itself according to these design specifications.

---

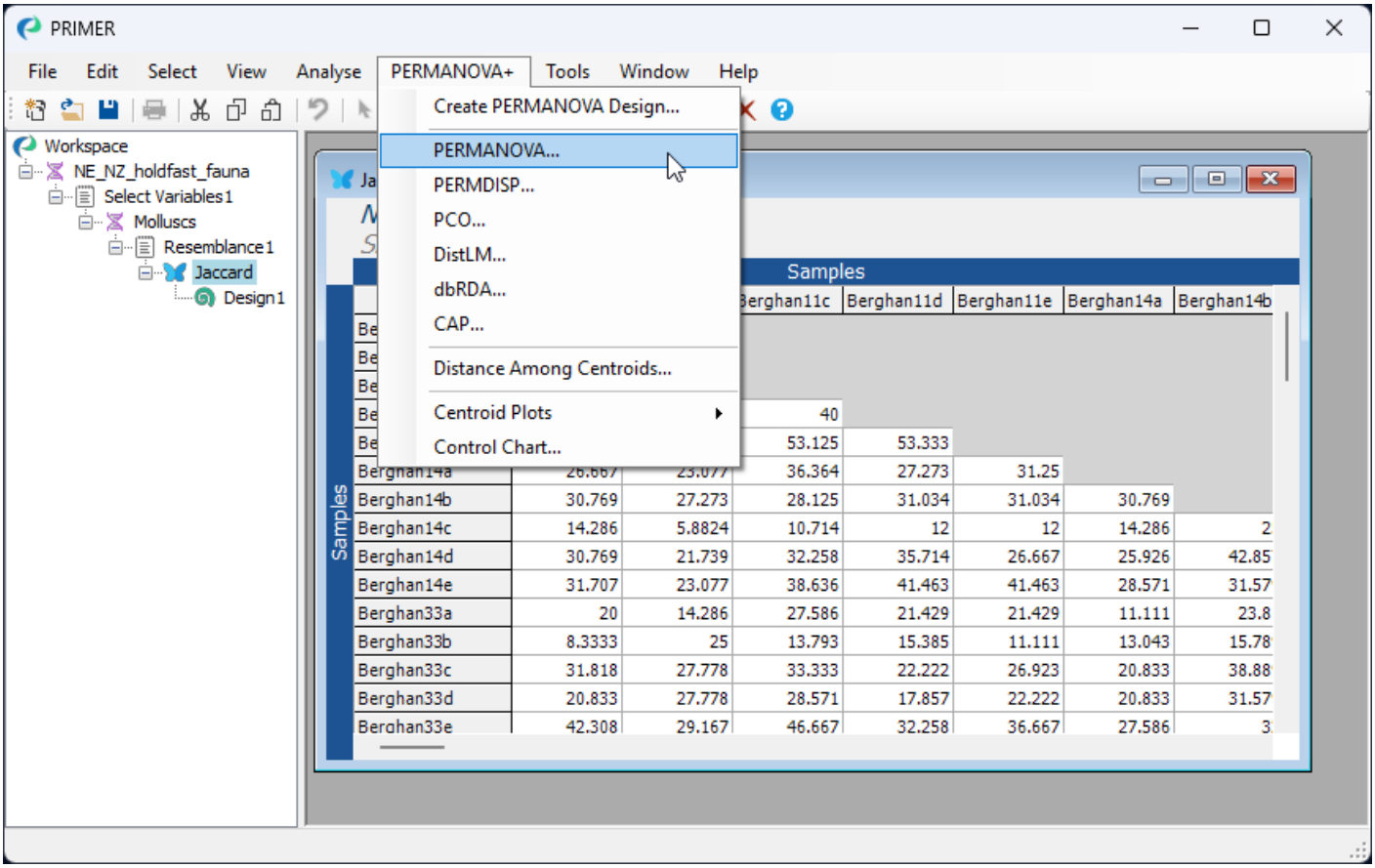
‡ *Note that a Design-file item in PRIMER 8 has its own properties, which you can see by clicking on Edit > Properties. Doing this shows a title for the design (which can be modified if desired) and you can also specify the number of factors (rows) in this way (rather than using the 'Add row' and 'Remove row' buttons):*


PRIMER
File
Edit
View
Tools
Window
Help
Clear Highlight
Cut Ctrl+X
Copy Ctrl+C
Paste Ctrl+V
Add Row
Insert Row
Delete Rows
Move Rows
Properties...
Design1
NE NZ kelp holdfast fauna
Add row
Remove row
Factor
Nested in
Type
Contrasts
Location
Random
Site
Location
Random
Area
Site
Random
PERMANOVA design properties
Title:
NE NZ kelp holdfast fauna
Number of factors: 3
OK
Cancel
Help
Dispersions
Allow for heterogeneity
Groups...
Term identifying groups with different dispersions:
Type III (partial)
Covariable-by-covariable
Row 3 Col 1

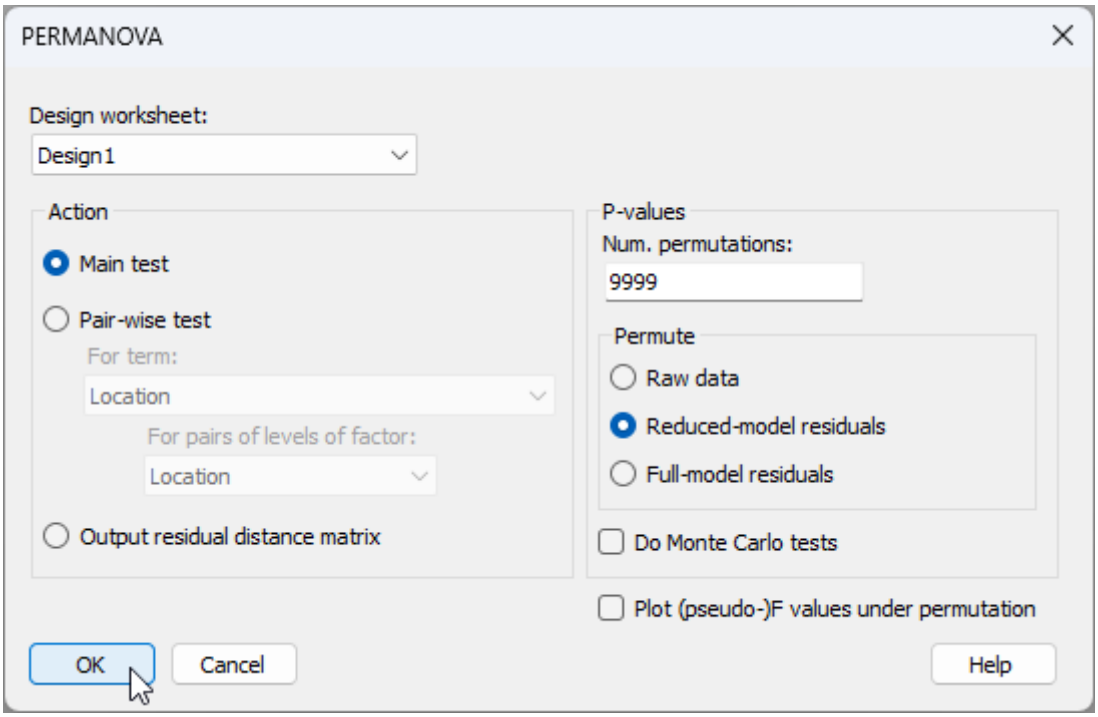
Step 4: Run PERMANOVA

Once the design file is created, we are ready to go ahead with the PERMANOVA analysis.

1. Click on the 'Jaccard' resemblance matrix in the Explorer tree so that it is the active item in the workspace, then click **PERMANOVA+** > **PERMANOVA...**



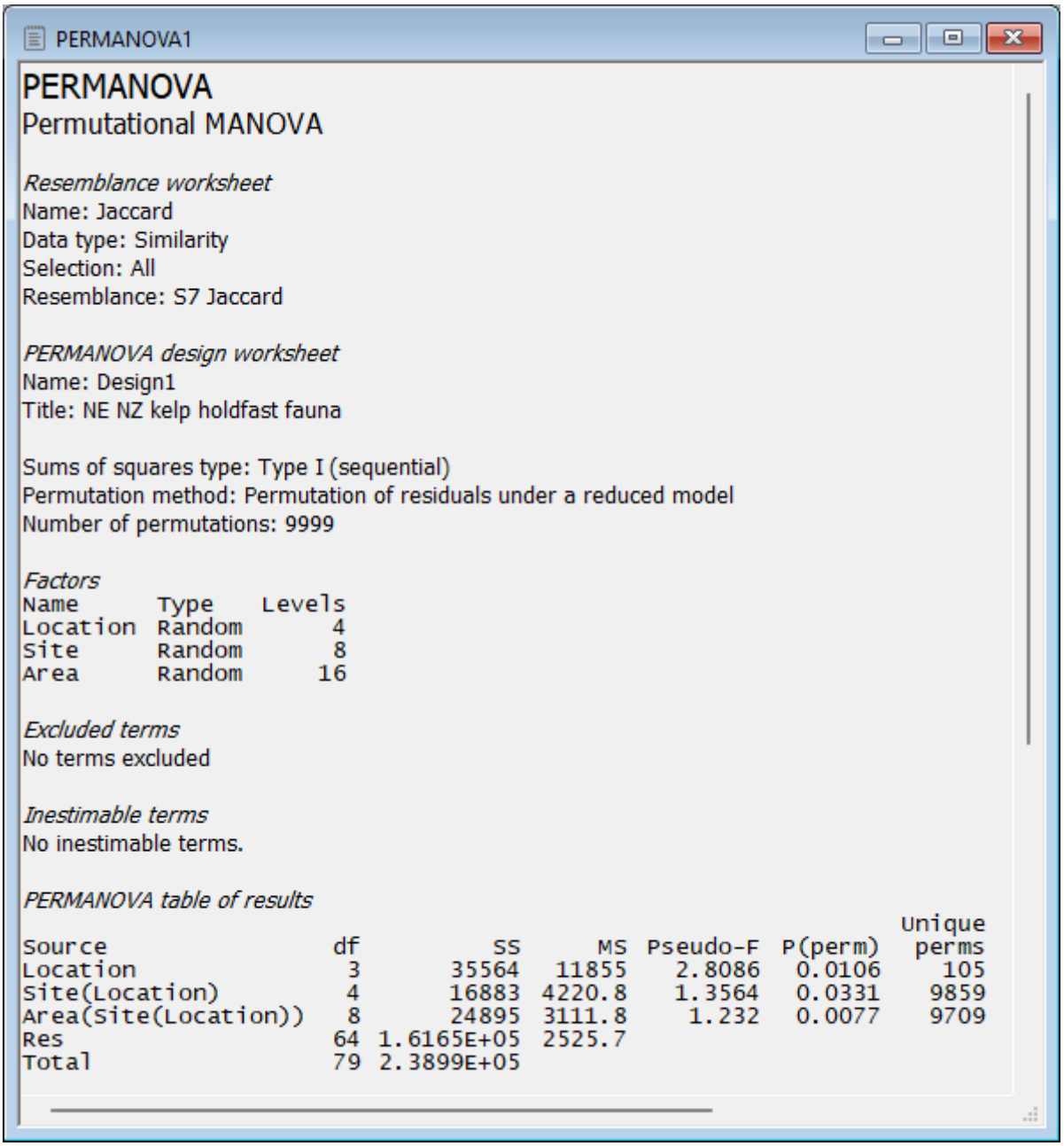
2. Check to see that the 'Design worksheet:' is Design1; this is the design file we created in the previous step that contains the three-way nested design. For the rest, we will keep the defaults in the PERMANOVA dialog, as shown below, then click **OK**.



3. This produces an output file (called 'PERMANOVA1') in the Explorer tree. It shows:

- the details of the choices you made in the PERMANOVA dialog to run the analysis;
- the details of your experimental design; and
- the PERMANOVA table of results

as follows:



PERMANOVA1

PERMANOVA
Permutational MANOVA

Resemblance worksheet
Name: Jaccard
Data type: Similarity
Selection: All
Resemblance: S7 Jaccard

PERMANOVA design worksheet
Name: Design1
Title: NE NZ kelp holdfast fauna

Sums of squares type: Type I (sequential)
Permutation method: Permutation of residuals under a reduced model
Number of permutations: 9999

Factors

Name	Type	Levels
Location	Random	4
Site	Random	8
Area	Random	16

Excluded terms
No terms excluded

Inestimable terms
No inestimable terms.

PERMANOVA table of results

Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Location	3	35564	11855	2.8086	0.0106	105
Site(Location)	4	16883	4220.8	1.3564	0.0331	9859
Area(Site(Location))	8	24895	3111.8	1.232	0.0077	9709
Res	64	1.6165E+05	2525.7			
Total	79	2.3899E+05				

Interpretation

These results show that there is statistically significant variability in the identities of molluscs among holdfasts at each of the three spatial scales in the experimental design: **Areas** ($F_{8,64}$ = 1.23, P < 0.01), **Sites** ($F_{4,8}$ = 1.36, P < 0.05) and **Locations** ($F_{3,4}$ = 2.81, P < 0.05). Note that the p-value for Locations is somewhat limited by the number of unique values of the pseudo-F statistic under permutation that are available here. Specifically, when we permute 2 samples per group (i.e., the 8 Sites) randomly across 4 groups (the Locations), there are just 105 unique values of pseudo-F that can be obtained, so the minimum possible p-value here is 1/105 = 0.0095).

Step 4 (continued): Key additional details about PERMANOVA in PRIMER

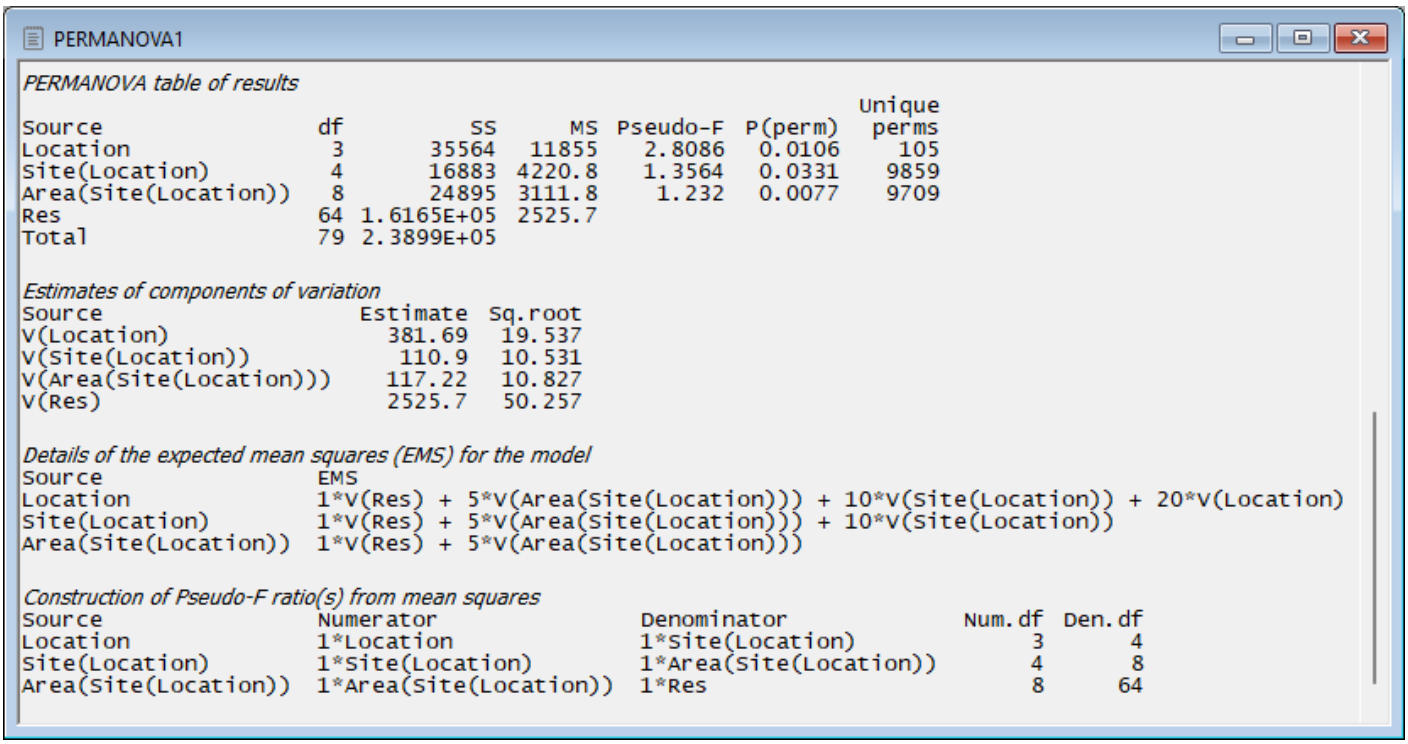
Following the PERMANOVA table of results, a suite of key additional details regarding the analysis can be seen in the PERMANOVA output file.

(*Note: It is not necessary to fully unpack all of these details to continue on with the analysis and interpretation of results, but some information is provided here to highlight what makes the implementation of PERMANOVA in PRIMER so unique, surpassing all other software tools in its robust handling of multi-factorial experimental designs).*

Additional details in the PERMANOVA output file include:

- estimates of the **components of variation** for each term in the model in the space of the resemblance measure.
- details of the **expected mean squares (EMS)** for each term in the model;
- the **construction of the pseudo-F ratios** for each term in the model from the appropriate mean squares (along with the associated numerator and denominator degrees of freedom); and

For example, scrolling down further in the output file from the PERMANOVA done on the holdfast data, we see:


PERMANOVA1

PERMANOVA table of results

Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms
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Site(Location)	4	16883	4220.8	1.3564	0.0331	9859
Area(Site(Location))	8	24895	3111.8	1.232	0.0077	9709
Res	64	1.6165E+05	2525.7			
Total	79	2.3899E+05				

Estimates of components of variation

Source	Estimate	Sq.root
V(Location)	381.69	19.537
V(Site(Location))	110.9	10.531
V(Area(Site(Location)))	117.22	10.827
V(Res)	2525.7	50.257

Details of the expected mean squares (EMS) for the model

Source	EMS
Location	1*V(Res) + 5*V(Area(Site(Location))) + 10*V(Site(Location)) + 20*V(Location)
Site(Location)	1*V(Res) + 5*V(Area(Site(Location))) + 10*V(Site(Location))
Area(Site(Location))	1*V(Res) + 5*V(Area(Site(Location)))

Construction of Pseudo-F ratio(s) from mean squares

Source	Numerator	Denominator	Num.df	Den.df
Location	1*Location	1*Site(Location)	3	4
Site(Location)	1*Site(Location)	1*Area(Site(Location))	4	8
Area(Site(Location))	1*Area(Site(Location))	1*Res	8	64

The implementation of PERMANOVA in PRIMER always uses expected mean squares (EMS) to construct correct tests for every term in the model; specifically:

- to construct the correct pseudo-F ratio; and

- to implement a permutation algorithm that
  - identifies the correct permutable units; and
  - accounts correctly for other terms in the model.

Of course, each term will require careful construction of its own test-statistic and its own permutation algorithm, both of which will depend on whether terms are fixed or random, whether there are other nested terms, covariates or interactions, etc. For unbalanced cases, the 'Type' of sum of squares is also important for the partitioning and correct construction of individual tests. All of these things can affect the EMS.

The EMS are also used to estimate the **components of variation** attributable to different sources of variation. These are *not* the same thing as raw R^2 values (as are typically used to compare the relative importance of individual predictor variables in regression models).† In PERMANOVA, these are calculated in a directly analogous way to the unbiased univariate ANOVA estimators. The column labeled 'Estimate' expresses these components in squared dissimilarity units, and its square root ('Sq.root', interpretable as a sort of standard deviation in the space of the resemblance measure) is also provided.

In the present example, we can see that the greatest variation occurs at the smallest spatial scale - from holdfast to holdfast within a given area (i.e., the square root of 'V(Res)' is 50.257 in Jaccard % dissimilarity units). The sources of variation (in order of importance, as quantified by the PERMANOVA model) are: Residual > Location > Area > Site.

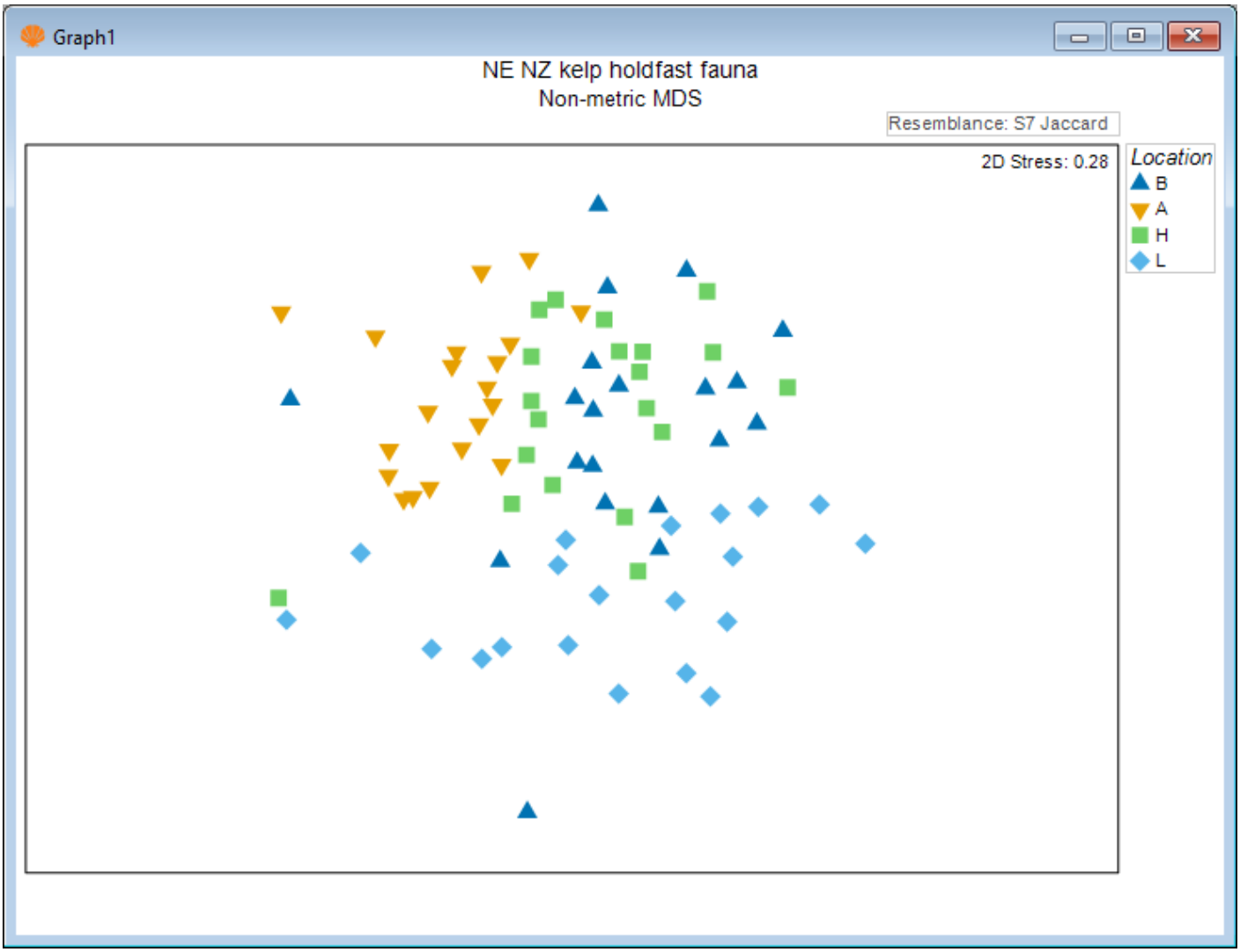
For more information about all of these key additional details provided in the PERMANOVA output file, please consult the PERMANOVA+ manual. For a discussion of why using R^2 values to compare the relative importance of terms in ANOVA models is inappropriate, see the section entitled 'What about R^2 values?' in the book: 'Should I use PRIMER or R?'.

Step 5: Ordination of centroids

Having seen the results of a PERMANOVA analysis, it is natural to wish to see a **visualisation** of the patterns among centroids belonging to different groups or combinations of levels of different factors in the study design. In many cases, particularly if there is a large number of replicates in a complex study design, if one were simply to create an ordination of all individual sampling units, there would just be a lot of noise (the plot may look very messy), due to high residual variation. This tends to obscure salient patterns and important effects.

Ordination of sampling units (all replicates)

We can produce a non-metric MDS ordination of the holdfast data by starting from the Jaccard resemblance matrix and clicking on **Analyse** > **MDS** > **Non-metric MDS...**, taking all of the defaults, then clicking **OK**. The resulting configurations are very unsatisfactory with quite high stress, either in 2 dimensions (stress = 0.28, shown below), or 3 dimensions (stress = 0.20).

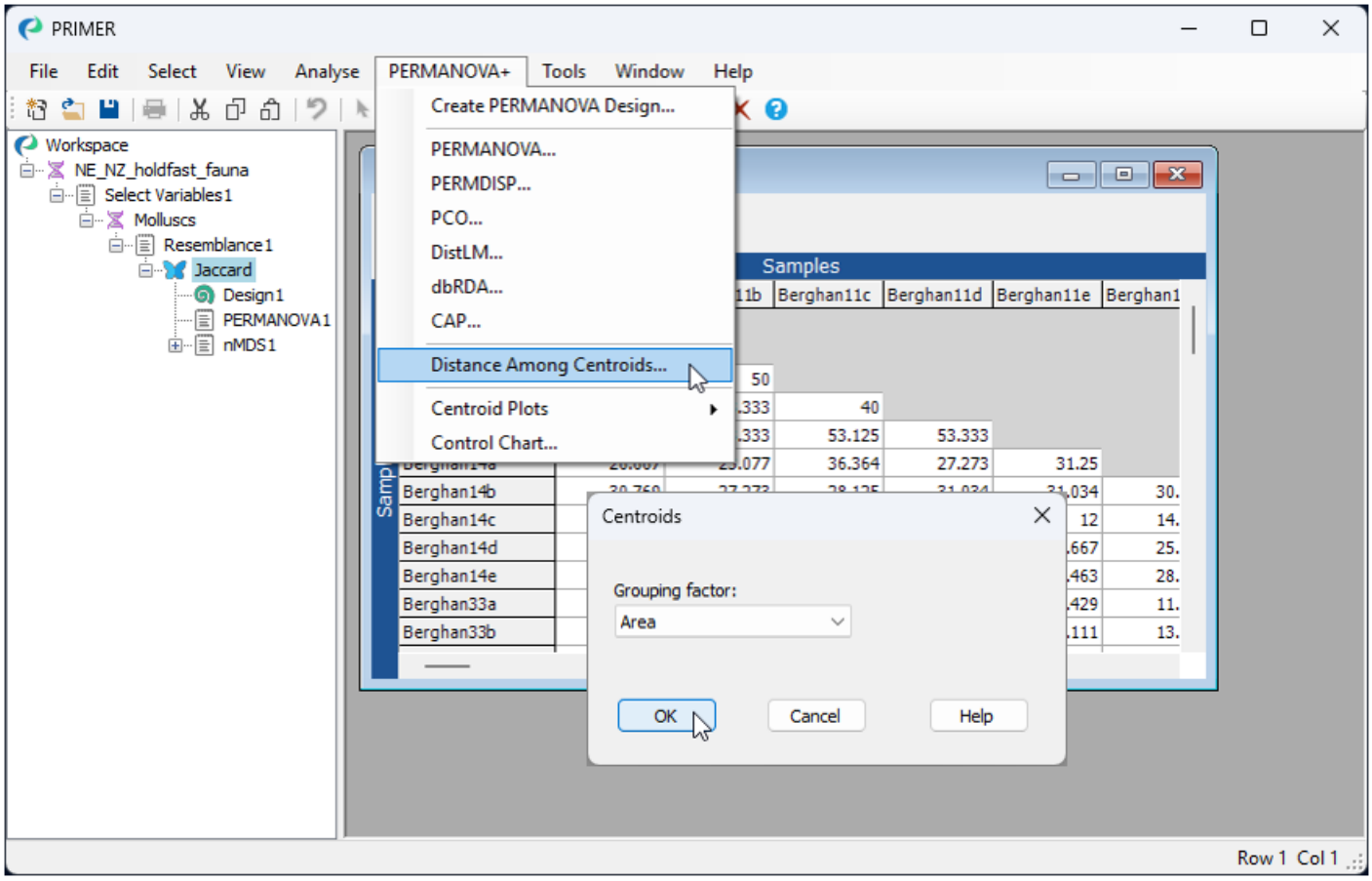


Seeing a bit of a 'mess' when we plot replicates like this is actually not too surprising in many cases, and particularly in this case, considering the very high variation (high turnover) in the identities of molluscs among holdfasts at small spatial scales (within areas), as seen on the previous page (recall that the residuals contributed by far the greatest source of variation to this system).

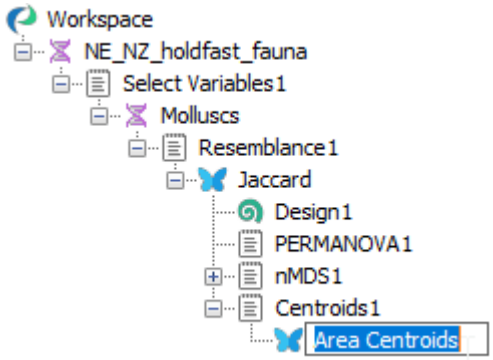
Ordination of distances among centroids

We can instead examine an ordination plot of the **centroids** in the space of the resemblance measure. In multi-factor designs when there is more than one factor and these factors are crossed with one another, we may need to created a single factor that consists of combinations of levels of the crossed factors (using **Edit** > **Factors...** > **Combine...**), but in the case of a fully nested design, and where nested factors all utilise unique labels (such as we have here), we can proceed without such a step.

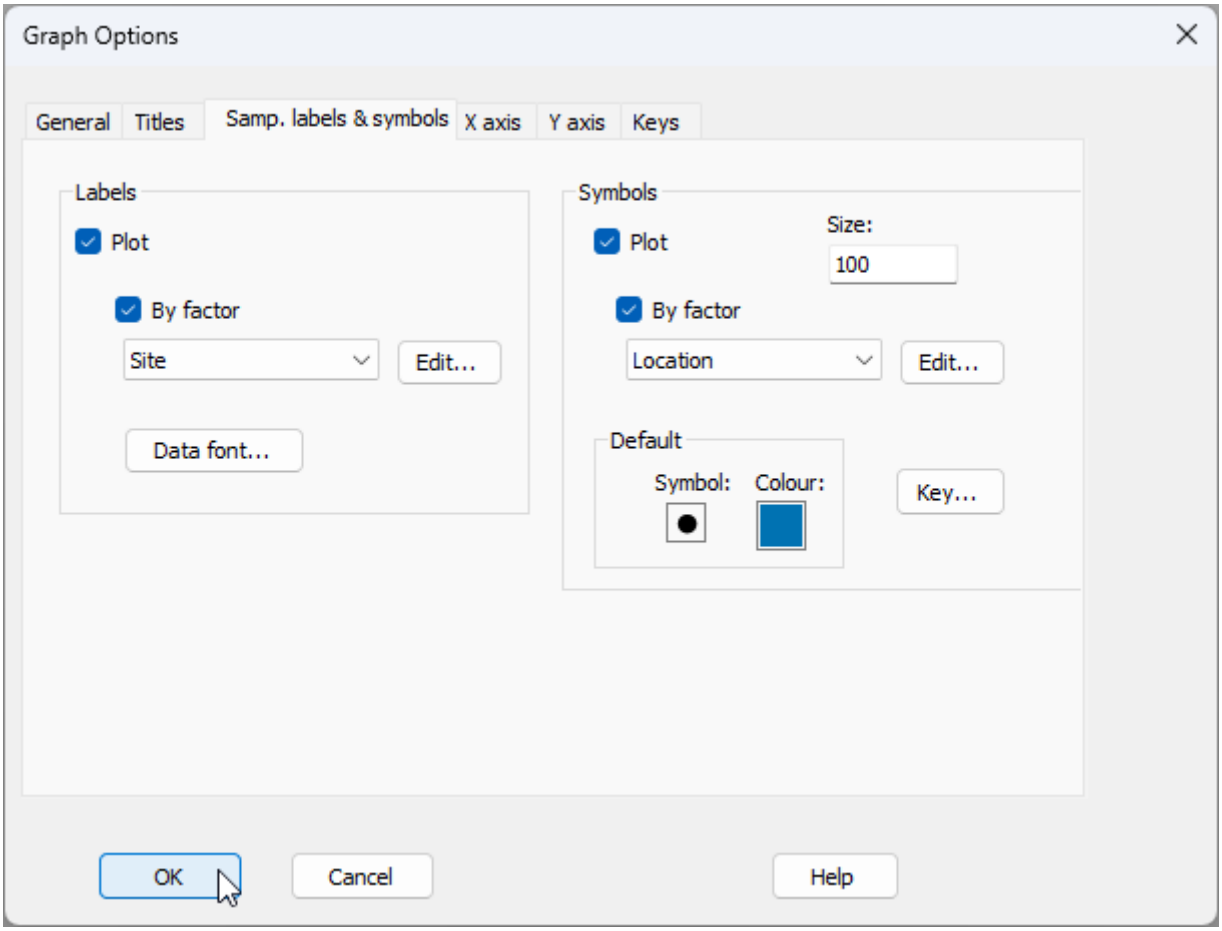
1. From the 'Jaccard' resemblance matrix, click **PERMANOVA+** > **Distances Among Centroids**, then in the 'Centroids' dialog, choose (Grouping factor: Area), and click **OK**.



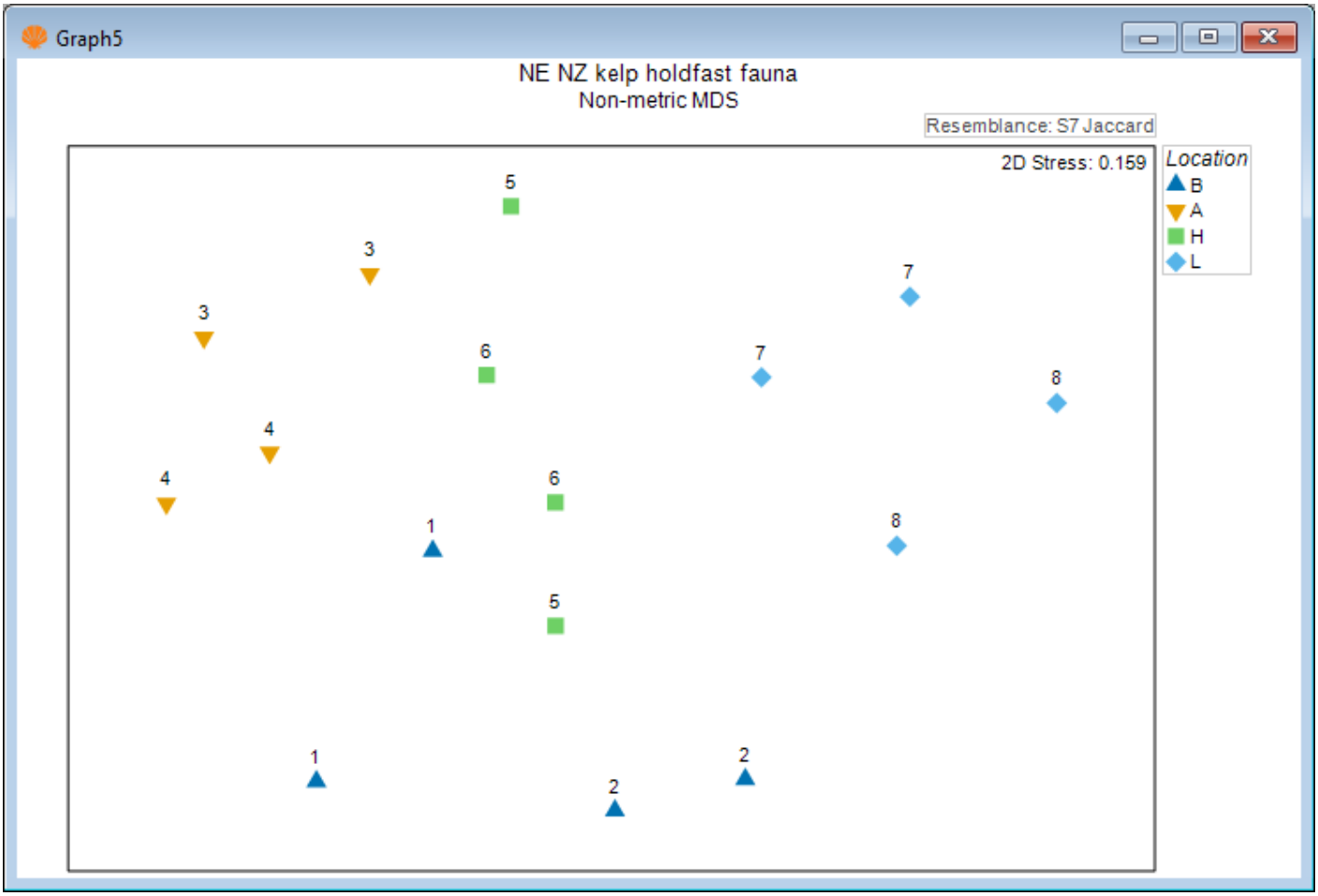
This will give you a matrix of Jaccard dissimilarities among the 16 Area centroids; each centroid being comprised of $n = 5$ replicate holdfasts within a given area. These are constructed in the space of the dissimilarity measure, which is not (quite) the same thing as calculating the arithmetic averages in the space of the original variables (i.e., that corresponds to a centroid in Euclidean space). In other words, these are the centroids just 'as PERMANOVA sees them' in *Jaccard* space, when doing the partitioning. You can re-name this matrix (from 'Resem1') to 'Area centroids'; i.e.,



2. From the 'Area centroids' resemblance matrix, click **Analyse** > **MDS** > **Non-metric MDS...**, take all the defaults and click **OK**.
3. From the 2D nMDS (probably called 'Graph5' in the Explorer tree), click on **Graph** > **Sample Labels & Symbols...**, then choose (Labels > $\checkmark$Plot > $\checkmark$By factor > Site) & (Symbols > $\checkmark$Plot > $\checkmark$By factor > Location), then click **OK**.



The result is a much more interpretable ordination plot, with far lower stress, *viz*:



Each point now represents the centroid (in Jaccard space) for n = 5 holdfasts in a given area. The numbers identify the 8 different sites, and the colours correspond to the 4 different locations. The patterns we see here are consistent with what was learned from the PERMANOVA analysis. More specifically, we can see that, within any particular location, the variation from one area to the next (any 2 centroids having the same symbol and number) is fairly similar to the variation between the two sites (any 2 centroids having the same colour, but a different number), and also that variation among locations (different colours) exceeds this - all four locations (colours) are clearly distinguishable from one another on the plot.

Summary of the PERMANOVA analysis

A summary of the essential steps associated with performing this PERMANOVA analysis of the holdfast data according to the 3-factor hierarchical experimental design is given in the table below:

Step	To implement in PRIMER:
1. Select **variable subset**	*From the original data sheet:* click **Select** > **Variables...** > ($\bullet$Indicator levels > Indicator name: Phylum), click **Levels...**> (Selection > Include: Mollusca), click **OK**.
2. Jaccard **resemblance**	*From the subset-selected data sheet:* click **Analyse** > **Resemblance** > (Measure $\bullet$Other > S7 Jaccard) & (Analyse between $\bullet$Samples), click **OK**.
3. Specify the **design**	• *From the resemblance matrix:* click **PERMANOVA+** > **Create PERMANOVA design...** • *Fill in the appropriate details of the design in the design file, including types of factors and any nested structures.*
4. Run **PERMANOVA**	*From the resemblance matrix:* click **PERMANOVA+** > **PERMANOVA...** > (Design Worksheet: Design1) & (default settings for the rest), click **OK**.
5. Ordination of **centroids**	• *From the resemblance matrix:* click **PERMANOVA+** > **Distance Among Centroids...** > (Grouping factor: Area), click **OK**. • *From the resulting resemblance matrix among area centroids:* click **Analyse** > **MDS** > **Non-metric MDS...**, take all the defaults and click **OK**. • *To specify labels and symbols:* click **Graph** > **Sample Labels & Symbols...**.