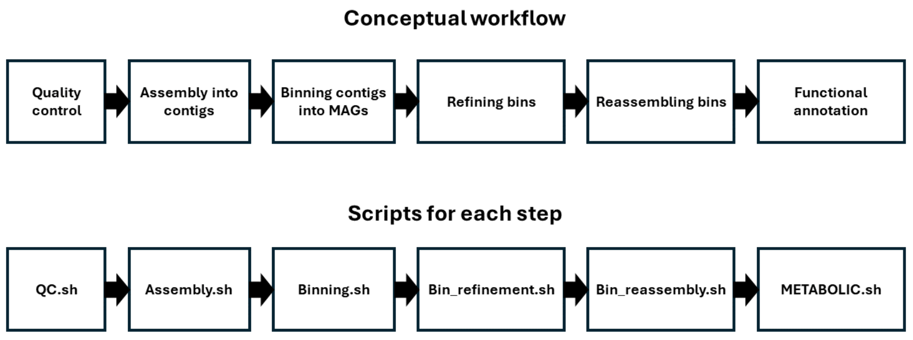
Recovery of Microbial Genomes from Metagenomes and their functional annotation

This workshop covers the recovery of microbial genomes from metagenomics samples using Metawrap and their functional annotation using METABOLIC



Notes on using these materials

- **Code can be copied from code blocks using the button at the top right of each code block with a single click**
- **To paste code into your terminal, you will need to right click.**
- **You can check the status of your running jobs using:** `bash squeue --me`

The first column of the output printed to the terminal will be the job identifier (JOBID). If you want to cancel your job, you can use: `bash scancel YOUR-JOB-ID` Obviously swapping 'YOUR-JOB-ID' for the actual job identifier.

- **If you lose connection or get lost at any point**

You can return to the directory we will be running all steps from using: `bash cd /uoa/scratch/users/"$USER"/UoA_Metagenomics_Workshop`

Setup

Environments

In order to ensure that all the packages we need and their dependencies are installed, and so that versions required by each of the programs we are running do not conflict with eachother, we will be using `conda` to manage our 'environments'.

What is an environment

An environment in Conda is a self-contained directory that contains a specific collection of packages and dependencies. This means you can have multiple environments on your system, each tailored for a particular project or task, without interfering with each other.

Managing environments with `conda` ensures: 1. Isolation: Different projects can require different versions of the same package. Environments keep these separate to avoid conflicts.

1. Reproducibility: By using environments, you can easily share the exact setup used for a project, ensuring others can replicate your work precisely.
2. Organization: Environments help you keep your workspace clean and organized, making it easier to manage projects and dependencies.

Environments for this workshop

In addition to installing the packages and their dependencies, MetaWRAP and METABOLIC are a bit complicated and rely on setting some paths to databases as well as running some setup scripts, which would take too long to do here. We will be using some pre-configured environments in this workshop that have everything you need to run these tools.

Load Miniconda3

Miniconda3 is a minimal installation of conda that is available on Maxwell as a module. We can load it using: `bash module load miniconda3`

We can then ensure that miniconda is available every time we log in by running: `bash conda init bash` and `bash source ~/.bashrc`

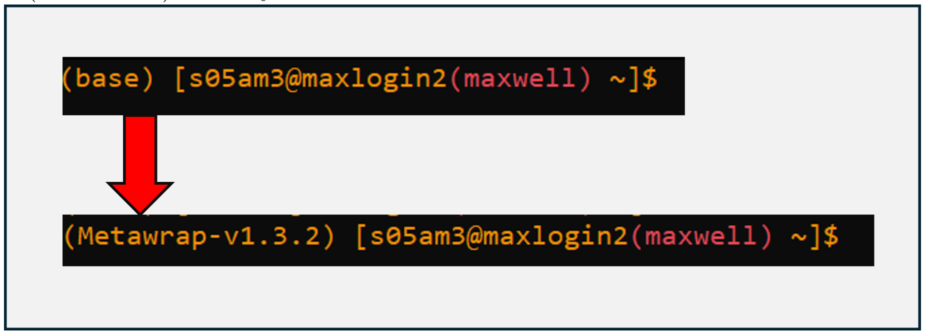
Note: If you already have a way of using conda on Maxwell that you are happy with, and you are confident enough to fix any conda-related issues that might arise during the use of the materials below, then feel free to skip the above steps.

Check the environments are set up correctly

Although we are providing pre-configured environments, we should check to make sure they are working properly for you and can find the databases required.

Check Metawrap environment

A Metawrap environment is provided for this training at `/uoa/scratch/shared/Soil_Microbiology_Group/Tra`
You can activate it using: `bash conda activate /uoa/scratch/shared/Soil_Microbiology_Group/Trainin`

You should see the environment (in brackets) before your username in the command prompt change as below: 

```
(base) [s05am3@maxlogin2(maxwell) ~]$
```

```
(Metawrap-v1.3.2) [s05am3@maxlogin2(maxwell) ~]$
```

Now we need to check that the paths to the databases are configured properly.
Verify that the output of: `bash echo $BLASTDB` matches:

/uoa/scratch/shared/Soil*Microbiology*Group/Training/Metagenomics/databases/NCBI_nt

and that the output of: `bash echo $TAXDUMP` matches:

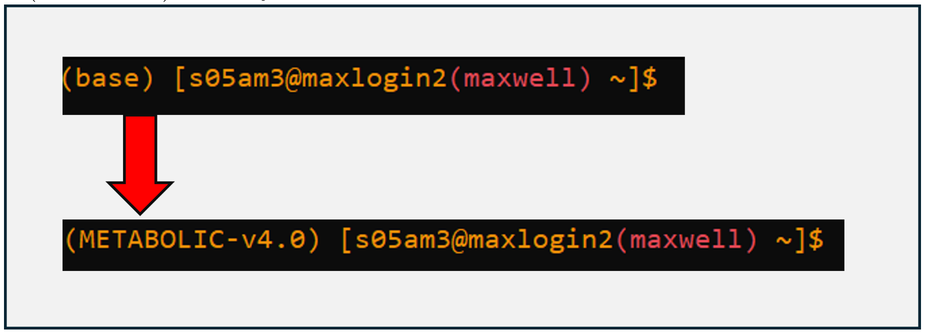
/uoa/scratch/shared/Soil*Microbiology*Group/Training/Metagenomics/databases/NCBI_tax

If it does, then Metawrap is ready to be used.

Deactivate the environment using: `bash conda deactivate`

Check METABOLIC environment

A Metawrap environment is provided for this training at `/uoa/scratch/shared/Soil_Microbiology_Group/Tra`
You can activate it using: `bash conda activate /uoa/scratch/shared/Soil_Microbiology_Group/Trainin`

You should see the environment (in brackets) before your username in the command prompt change as below: 

```
(base) [s05am3@maxlogin2(maxwell) ~]$
```

```
(METABOLIC-v4.0) [s05am3@maxlogin2(maxwell) ~]$
```

Now we need to check that the paths to the databases are configured properly.
Verify that the output of: `bash echo $GTDBTK_DATA_PATH` matches:

> /uoa/scratch/shared/Soil*Microbiology*Group/Training/Metagenomics/databases/GTDB-Tk_r220

and the output of: `bash echo $GTDB_DATA_PATH` matches:

> /uoa/scratch/shared/Soil*Microbiology*Group/Training/Metagenomics/databases/GTDB-Tk_r220

If it does, then METABOLIC is ready to be used.

Deactivate the environment using: `bash conda deactivate`

Clone (copy) this repository to your own space on Maxwell

This will give you environment files and the scripts needed to run the analysis (which will be uploaded AFTER the workshop, to force you to write them yourself for now.)

Navigate to somewhere sensible: `bash cd /uoa/scratch/users/"$USER"` *Note: you do not need to change "$USER" here, this will automatically use your correct username.*

Clone the repository using: `bash git clone https://github.com/biomoff/UoA_Metagenomics_Workshop`

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0. Get the Raw Data

A small metagenomics dataset has been prepared for this workshop. Navigate to the workshop folder `bash cd /uoa/scratch/users/"$USER"/UoA_Metagenomics_Workshop`

and link the raw data: `bash ln -s /uoa/scratch/shared/Soil_Microbiology_Group/Training/Metagenomi` `0_RAW-READS`

You should now have a directory called `0_RAW-READS` containing some metagenomics sequence files:

> A*1.fastq*
> *A*2.fastq
> B*1.fastq*
> *B*2.fastq

These are the forward (`_1`) and reverse (`_2`) reads for 2 metagenome samples (`A` and `B`). You can imagine these as being, for example, 2 different soil samples or samples from 2 different patients.

Verify that what you have matches the above: `bash ls -l 0_RAW-READS/`

If it matches, you can now begin the pipeline, starting with some quality control

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1. Quality Control using Metawrap's Read QC module

Before we can proceed with any sort of analysis, we need to do some quality control on the data we have.

We will be using the `read_qc` module of Metawrap to trim the reads. Often you might want to remove reads from a host (e.g. human), but we will be skipping that step here for simplicity as it is not required in our dataset. If you need to do it on your own data, you can find the information here.

Making the QC.sh submission script

We need to create the `QC.sh` script to run read trimming on our two samples. Start by creating and opening this file in the `Scripts` subdirectory: `bash module load nano nano Scripts/QC.sh`

You should now be inside an open empty file, which we will gradually add to in order to build up our submission script for read QC.

Adding Slurm scheduler parameters

We need to tell the Slurm scheduler how much resource we want to allocate to the job. The following parameters have been optimised for this dataset. Add them to your open `QC.sh` file by copying and pasting: “‘bash

!/bin/bash

SBATCH --partition=uoa-compute

SBATCH --time=01:00:00

SBATCH --cpus-per-task=2

SBATCH --mem-per-cpu=2G

SBATCH --mail-type=ALL

SBATCH --mail-user=

“`*note: add in your email address after`mail-user=‘ in order to get email updates for the job*

Adding some description of the version the script was written for

It is also good practice to note which version of the package you are using the script was written for, in the event that a newer version becomes available. You can add something like the below which will be useful if you use the script later or send it to somebody else to use. Copy and paste it into your open file: “‘bash

This was written for metawrap version 1.3.2

“‘

Adding code for loading miniconda and activating the correct environment

Next, we need to make sure that our job is using the environment that we made, so that it has access to Metawrap. Add the below to your open file: “‘bash

Source miniconda3 installation and activate environment

source /opt/software/uoa/apps/miniconda3/latest/etc/profile.d/conda.sh conda activate /uoa/scratch/shared/Soil*Microbiology*Group/Training/Metagenomics/envs/Metawrap-v1.3.2

“‘

Adding code to set the input and output directories

It is also useful to be able to see at the top of the file what the input and output directories that the script will look for files in / output files to. This means that if you want to change the name of the output directory you only need to do it once at the top by changing the variable, and it will automatically apply to the whole script. Add the below to your open file: “‘bash

Set directory and file name variables

InputDir=0*RAW-READS OutputDir=1*READ-QC

“‘ *note: avoid changing the names here, as the rest of this workshop will reference these directories specifically.*

Add code to make the output directories

“‘bash

Make output directories

mkdir -p "$OutputDir"

“‘

Adding the code to run Metawrap's QC module

Finally you can add the code to use Metawrap to perform read trimming on your samples: "'bash

QC and trim raw reads:

metawrap read*qc -1 "$InputDir"/A*1.fastq -2 "$InputDir"/A*2.fastq -t 2 -o "$OutputDir"/A --skip-bmtagger metawrap read*qc -1 "$InputDir"/B*1.fastq -2 "$InputDir"/B*2.fastq -t 2 -o "$OutputDir"/B --skip-bmtagger

"'

This will read in the forward reads (`-1`) and the reverse reads (`-2`) from your samples, use 2 cpu threads (`-t 2`) to perform the read QC, and then send the output files to your specified output directory (`-o`). We have used the `--skip-bmtagger` flag to skip removal of host sequences.

Save the file by using Ctrl+o, hit Enter, then close the file using Ctrl+x.

Running the QC.sh script

You can now run the script as below: `bash sbatch Scripts/QC.sh`

This should take roughly 20-25 minutes to run.

If it does not run within the allocated time, view your job using `squeue --me` to find the JOBID, and cancel the job using `scancel JOBID`.

remove the incomplete output folder using: `bash rm -rf 1_READ-QC`

and link to the example output provided using: `bash ln -s /uoa/scratch/shared/Soil_Microbiology_Grou` `1_READ-QC`

Inspecting the output of Metawrap's QC module

You should, once it finishes running, have an output directory called `1_READ-QC` that contains two subdirectories, `A` and `B`. These will each contain 2 files `final_pure_reads_1.fastq` and `final_pure_reads_2.fastq` for the trimmed forward and reverse reads respectively, and some output directories with QC reports: `pre-QC_report` and `post-QC_report`. Verify that you have these outputs using: `bash ls -l 1_READ-QC/A ls -l 1_READ-QC/B`

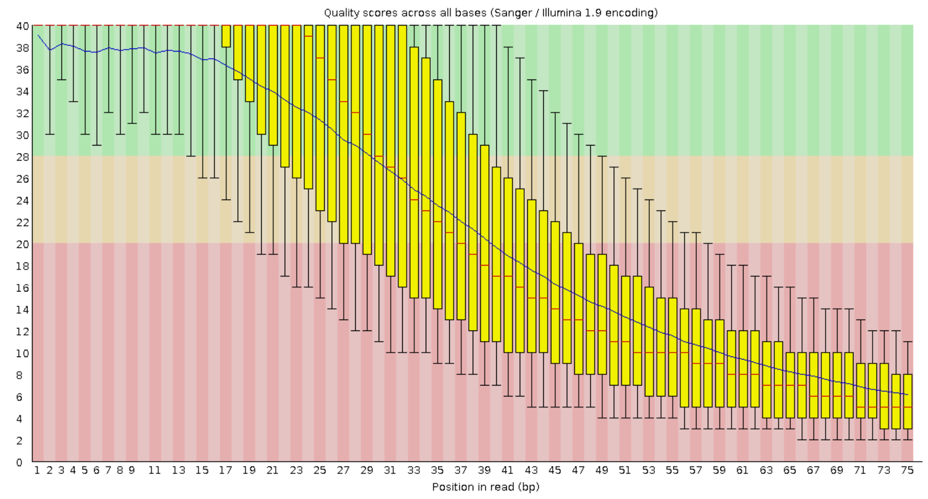
Within `pre-QC_report` and `post-QC_report` you would find:

> final*pure*reads*1*fastqc.html
> final*pure*reads*2*fastqc.html

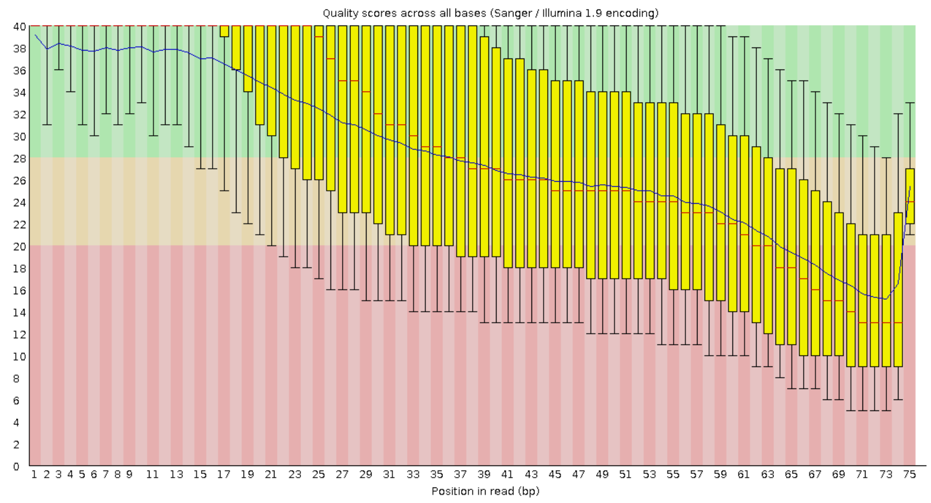
These QC report HTML files can be inspected by copying them over to your own machine (using WinSCP or other methods) and viewing them in a browser.

We will skip that with our data for now, but below is an example of what you might expect to see:

pre-QC reads:



post-QC reads:



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2. Assembling the reads into Contigs using Metawrap's Assembly module

Now that we have high quality reads, we need to assemble them into contigs using Metawrap's `assembly` module.

Depending on your study, you might want to assemble the reads from all samples together, or separately. If, for example, you have multiple replicates from a single sample (e.g. a particular soil), it may be of use to coassemble all those samples together. In our case we will stick with assembling our samples individually.

Metawrap offers 2 different assembly algorithms, MetaSPADES and Megahit. MetaSPADES is usually recommended for all but large datasets, but we will use Megahit here to reduce computational burden.

Making the Assembly.sh submission script

We need to create the `Assembly.sh` script to assemble our two samples. Start by creating and opening this file in the `Scripts` subdirectory: `bash nano Scripts/Assembly.sh`

You should now be inside an open empty file, which we will gradually add to in order to build up our submission script for assembly of reads into contigs.

Adding Slurm scheduler parameters

We need to tell the Slurm scheduler how much resource we want to allocate to the job. The following parameters have been optimised for this dataset. Add them to your open `Assembly.sh` file by copying and pasting: “‘bash

!/bin/bash

SBATCH --partition=uoa-compute

SBATCH --time=01:00:00

SBATCH --cpus-per-task=16

SBATCH --mem-per-cpu=250M

SBATCH --mail-type=ALL

SBATCH --mail-user=

“`*note: add in your email address after`mail-user=‘ in order to get email updates for the job*

Adding some description of the version the script was written for

It is also good practice to note which version of the package you are using the script was written for, in the event that a newer version becomes available. You can add something like the below which will be useful if you use the script later or send it to somebody else to use. Copy and paste it into your open file: “‘bash

This was written for metawrap version 1.3.2

“‘

Adding code for loading miniconda and activating the correct environment

Next, we need to make sure that our job is using the environment that we made, so that it has access to Metawrap. Add the below to your open file: “‘bash

Source miniconda3 installation and activate environment

source /opt/software/uoa/apps/miniconda3/latest/etc/profile.d/conda.sh conda activate /uoa/scratch/shared/Soil*Microbiology*Group/Training/Metagenomics/envs/Metawrap-v1.3.2

“‘

Adding code to set the input and output directories

As above, add the below to your open file: “‘bash

Set directory and file name variables

InputDir=1*READ-QC OutputDir=2*ASSEMBLY

“‘ *note: avoid changing the names here, as the rest of this workshop will reference these directories specifically.*

Add code to make the output directories

“‘bash

Make output directories

mkdir -p "$OutputDir"/A mkdir -p "$OutputDir"/B

“‘

Add code to assemble the reads into contigs using Metawrap's Assembly module

We want to run the assembly module individually on each sample, so we will do that by adding the following: “‘bash

Assemble contigs for sample A using metawrap assembly:

metawrap assembly -1 "$InputDir"/A/final*pure*reads*1.fastq -2 "$InputDir"/A/final*pure*reads*2.fastq -m 4 -t 16 --megahit -o "$OutputDir"/A

Assemble contigs for sample B using metawrap assembly:

metawrap assembly -1 "$InputDir"/B/final*pure*reads*1.fastq -2 "$InputDir"/B/final*pure*reads*2.fastq -m 4 -t 16 --megahit -o "$OutputDir"/B

"‘

This will take in the trimmed forward reads `-1` and the trimmed reverse reads `-2`, tell the assembler how much memory we have allocated `-m 4` (4 GB) and how many CPU threads to use `-t 16`, to use the megahit assembly algorithm `--megahit`, and where to send the output `-o`. There is the option to change the minimum length of assembled contigs to output, `-l`, but we will leave it at the default of 1000 bp.

Save the file by using Ctrl+o, hit Enter, then close the file using Ctrl+x.

Running the Assembly.sh script

You can now run the script as below: `bash sbatch Scripts/Assembly.sh`

It should take about 25-30 minutes to run.

If it does not run within the allocated time, view your job using `squeue --me` to find the JOBID, and cancel the job using `scancel JOBID`.

remove the incomplete output folder using: `bash rm -rf 2_ASSEMBLY`

and link to the example output provided using: `bash ln -s /uoa/scratch/shared/Soil_Microbiology_Grou` `2_ASSEMBLY`

Inspecting the output of Metawrap's assembly module

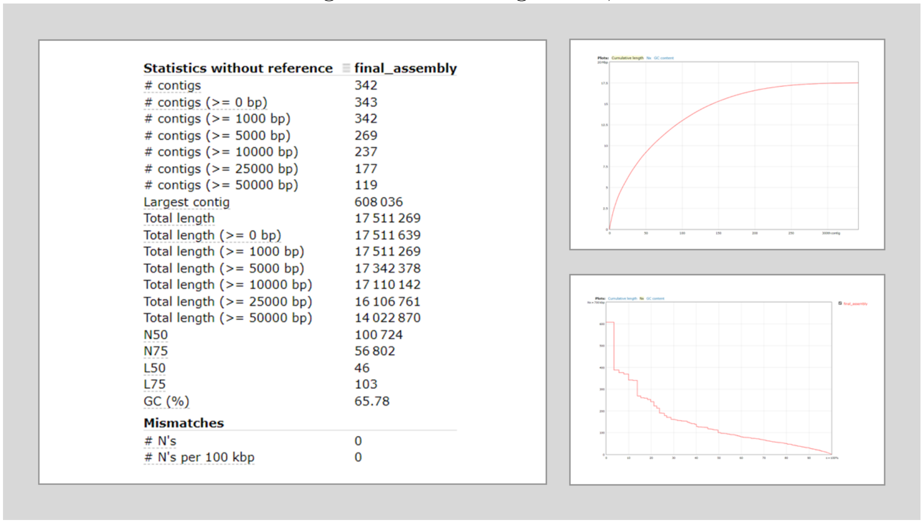
Once `Assembly.sh` has finished running, you should have a subdirectory called `2_ASSEMBLY` that contains subdirectories `A` and `B`. Each of these will contain 2 output files and 2 directories containing intermediate files:

QUAST*out*
*assembly*report.html
final_assembly.fasta
megahit

`megahit` and `QUAST_out` contain intermediate files. `final_assembly.fasta` is the final assembly file containing the contigs and `assembly_report.html` is the assembly report generated by QUAST.

Verify that you have these files for both samples A and B by running: `bash ls -l 2_ASSEMBLY/A ls -l 2_ASSEMBLY/B`

The full assembly report can be inspected by transferring it to your own machine (using WinSCP or other methods) and viewing it in a browser. The report will contain some statistics about the assembly as well as some handy plots of cumulative assembly size by number of contigs, and the size of the contigs in descending order, as well as GC content:



Statistics without reference	final_assembly
# contigs	342
# contigs (>= 0 bp)	343
# contigs (>= 1000 bp)	342
# contigs (>= 5000 bp)	269
# contigs (>= 10000 bp)	237
# contigs (>= 25000 bp)	177
# contigs (>= 50000 bp)	119
Largest contig	608 036
Total length	17 511 269
Total length (>= 0 bp)	17 511 639
Total length (>= 1000 bp)	17 511 269
Total length (>= 5000 bp)	17 342 378
Total length (>= 10000 bp)	17 110 142
Total length (>= 25000 bp)	16 106 761
Total length (>= 50000 bp)	14 022 870
N50	100 724
N75	56 802
L50	46
L75	103
GC (%)	65.78
Mismatches	
# N's	0
# N's per 100 kbp	0

However, we will just quickly inspect the output here on the command line. Copy and paste the below into the command line: `bash tail -n 9 2_ASSEMBLY/A/QUAST_out/report.txt tail -n 9 2_ASSEMBLY/B/QUAST_out/report.txt`

You should get an output that looks like this for sample A:

```
# contigs 345
Largest contig 608195
Total length 17514579
GC (%) 65.77
N50 100354
N75 56802
L50 47
L75 105
# N's per 100 kbp 0.00
```

and like this for sample B:

```
# contigs 355
Largest contig 393717
Total length 16614804
GC (%) 63.61
N50 93956
N75 54588
L50 55
L75 113
# N's per 100 kbp 0.00
```

Now that we have assembled our reads into contigs, it is time to

bin the contigs into different bins to make Metagenome Assembled Genomes (MAGs)

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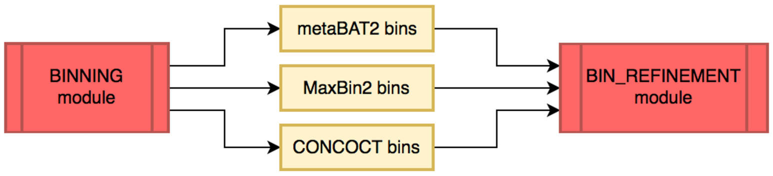
3. Binning into MAGs using Metawrap's Binning module

Binning is a crucial step in assembling MAGs from metagenomics data, and involves the placing of contigs into different bins based on taxonomy-informed or taxonomic indepedent methods. Taxonomic indepedent methods use GC content, K-mer frequencies, read depth, and co-variation of abundance across different samples to assign contigs to bins. Here we will be using Maxbin2, which uses tetranucleotide frequences, and MetaBAT2, which uses a graph-based approach on contig similarity.

Note: It is useful to remember that these bins often represent a collection of closely related taxa. 1 MAG != 1 genome from 1 organism.

Metawrap uses multiple different binning algorithms and combines their output to



yield more accurate bins:

We will now write the submission script to run Metawrap's Binning module.

Making the Binning.sh submission script

We need to create the `Binning.sh` script to assemble our two samples. Start by creating and opening this file in the `Scripts` subdirectory: `bash nano Scripts/Binning.sh`

You should now be inside an open empty file, which we will gradually add to in order to build up our submission script for assigning contigs to different bins.

Adding Slurm scheduler parameters

We need to tell the Slurm scheduler how much resource we want to allocate to the job. The following parameters have been optimised for this dataset. Add them to your open `Binning.sh` file by copying and pasting: “‘bash

!/bin/bash

SBATCH --partition=uoa-compute

SBATCH --time=01:00:00

SBATCH --cpus-per-task=4

SBATCH --mem-per-cpu=1G

SBATCH --mail-type=ALL

SBATCH --mail-user=

“`*note: add in your email address after`mail-user=‘ in order to get email updates for the job*

Adding some description of the version the script was written for

It is also good practice to note which version of the package you are using the script was written for, in the event that a newer version becomes available. You can add something like the below which will be useful if you use the script later or send it to somebody else to use. Copy and paste it into your open file: “‘bash

This was written for metawrap version 1.3.2

“‘

Adding code for loading miniconda and activating the correct environment

Next, we need to make sure that our job is using the environment that we made, so that it has access to Metawrap. Add the below to your open file: “‘bash

Source miniconda3 installation and activate environment

source /opt/software/uoa/apps/miniconda3/latest/etc/profile.d/conda.sh conda activate /uoa/scratch/shared/Soil*Microbiology*Group/Training/Metagenomics/envs/Metawrap-v1.3.2

“‘

Adding code to set the input and output directories

As above, add the below to your open file: “‘bash

Set directory and file name variables

Reads=1*READ-QC Assembly=2*ASSEMBLY OutputDir=3_INITIAL-BINNING

"' *note: avoid changing the names here, as the rest of this workshop will reference these directories specifically.*

Add code to make the output directories

"'bash

Make output directories

mkdir -p "$OutputDir"/A mkdir -p "$OutputDir"/B

"'

Add code to assign the contigs to bins using Metawrap's binning module

We want to run the assembly module individually on each sample, so we will do that by adding the following: "'bash

Bin contigs using Metawrap binning for sample A

metawrap binning -o "$OutputDir"/A -t 4 -a "$Assembly"/A/final_assembly.fasta --metabat2 --maxbin2 --universal "$Reads"/A/*_1.fastq "$Reads"/A/*_2.fastq

Bin contigs using Metawrap binning for sample B

metawrap binning -o "$OutputDir"/B -t 4 -a "$Assembly"/B/final_assembly.fasta --metabat2 --maxbin2 --universal "$Reads"/B/*_1.fastq "$Reads"/B/*_2.fastq

"'

Here we are using Metawrap's `binning` module to bin contigs, specifying the number of threads (`-t 4`), the location of the assembly file (`-a`), which binning algorithms to use (`--metabat2 --maxbin2`), and to use universal marker genes rather than just bacterial marker genes (`--universal`) which improves archaeal binning. We then specify that path to the raw reads as positional arguments at the end. There are other options that can be set, but we have left those with the defaults. For your own data, you can check them using `metawrap binning --help`.

Save the file by using Ctrl+o, hit Enter, then close the file using Ctrl+x.

Running the Binning.sh script

You can now run the script as below: `bash sbatch Scripts/Binning.sh`

It should take about 15-20 minutes to run.

If it does not run within the allocated time, view your job using `squeue --me` to find the JOBID, and cancel the job using `scancel JOBID`.

remove the incomplete output folder using: `bash rm -rf 3_INITIAL-BINNING`

and link to the example output provided using: `bash ln -s /uoa/scratch/shared/Soil_Microbiology_Group` `3_INITIAL-BINNING`

Inspecting the output of Metawrap's Binning module

You should now have a directory called `3_INITIAL-BINNING` that contains subdirectories `A` and `B`, which will each contain a subdirectory for each of the binning methods and a subdirectory with intermediate files:

> maxbin2*bins*
> *metabat2*bins
> work_files

Verify you have these using: `bash ls -l 3_INITIAL-BINNING/A ls -l 3_INITIAL-BINNING/B`

Within each of these you should have the fasta format files of the various bins contigs have been assigned to, e.g. for sample A: `bash ls -l 3_INITIAL-BINNING/A/maxbin2_bins`

> bin.0.fa
> bin.1.fa
> bin.2.fa

`bash ls -l 3_INITIAL-BINNING/A/metabat2_bins`

> bin.1.fa
> bin.2.fa
> bin.3.fa
> bin.unbinned.fa

We will refine these in the next step to consolidate the results of the 2 different binners.

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4. Refining bins using Metawrap's Bin Refinement module

As we mentioned above, Metawrap uses multiple binners and then combines their output to create consolidated bins that are better than those produced by any single algorithm. We will use Metawrap's `bin_refinement` module to do this.

Making the .sh submission script

We need to create the `Bin_refinement.sh` script to assemble our two samples. Start by creating and opening this file in the `Scripts` subdirectory: `bash nano Scripts/Bin_refinement.sh`

You should now be inside an open empty file, which we will gradually add to in order to build up our submission script for the refinement of the various bin sets.

Adding Slurm scheduler parameters

We need to tell the Slurm scheduler how much resource we want to allocate to the job. The following parameters have been optimised for this dataset. Add them to your open `Bin_refinement.sh` file by copying and pasting: “‘bash

!/bin/bash

SBATCH --partition=uoa-compute

SBATCH --time=02:00:00

SBATCH --cpus-per-task=4

SBATCH --mem-per-cpu=12G

SBATCH --mail-type=ALL

SBATCH --mail-user=

“`*note: add in your email address after`mail-user=‘ in order to get email updates for the job*

Adding some description of the version the script was written for

It is also good practice to note which version of the package you are using the script was written for, in the event that a newer version becomes available. You can add something like the below which will be useful if you use the script later or send it to somebody else to use. Copy and paste it into your open file: “‘bash

This was written for metawrap version 1.3.2

“‘

Adding code for loading miniconda and activating the correct environment

Next, we need to make sure that our job is using the environment that we made, so that it has access to Metawrap. Add the below to your open file: “‘bash

Source miniconda3 installation and activate environment

source /opt/software/uoa/apps/miniconda3/latest/etc/profile.d/conda.sh conda activate /uoa/scratch/shared/Soil*Microbiology*Group/Training/Metagenomics/envs/Metawrap-v1.3.2

“‘

Adding code to set the input and output directories

As above, add the below to your open file: “‘bash

Set directory and file name variables

Reads=1*READ-QC* *Bins=3*INITIAL-BINNING OutputDir=4_BIN-REFINEMENT

“‘ *note: avoid changing the names here, as the rest of this workshop will reference these directories specifically.*

Add code to make the output directories

“‘bash

Make output directories

mkdir -p "$OutputDir"/A mkdir -p "$OutputDir"/B

“‘

Add code to refine the bins from different methods using Metawrap's bin_refinement module

We want to run the assembly module individually on each sample, so we will do that by adding the following: “‘bash

Run Metawrap's bin refinement module for sample A

metawrap bin*refinement -o "$OutputDir"/A -t 4 -A "$Bins"/A/metabat2*bins/ -B "$Bins"/A/maxbin2_bins/ -c 50 -x 10

Run Metawrap's bin refinement module for sample B

metawrap bin*refinement -o "$OutputDir"/B -t 4 -A "$Bins"/B/metabat2*bins/ -B "$Bins"/B/maxbin2_bins/ -c 50 -x 10

“‘

Here, we are using Metawrap's `bin_refinement` module to take in the multiple bin sets and consolidate them into final improved bins. Here we are only using MetaBAT2 (`-A`) and Maxbin2 (`-B`) bin sets to consolidate. If we had run binning with CONCOCT, we could also supply that using `-C <path-to-concoct-bins>`. It is actually possible to supply bins produced using anything binning algorithm you like. Consult the documentation if this is something you want to do with your own data. We have also specified the number of threads to use (`-t 4`), and where to send the output (`-o`). Finally, we have used the `-c` and `-x` flags to set thresholds for completeness and contamination, which can be tailored for your own data depending on your needs. This uses the proportion of expected single copy genes that are present (completeness), and the proportion of expected single copy genes that are present more than once (contamination) in each bin.

Save the file by using Ctrl+o, hit Enter, then close the file using Ctrl+x.

Running the Bin_refinement.sh script

You can now run the script as below: `bash sbatch Scripts/Bin_refinement.sh`

If it does not run within the allocated time, view your job using `squeue --me` to find the JOBID, and cancel the job using `scancel JOBID`.

remove the incomplete output folder using: `bash rm -rf 4_BIN-REFINEMENT`

and link to the example output using: `bash ln -s /uoa/scratch/shared/Soil_Microbiology_Group/Traini` `4_BIN-REFINEMENT`

Inspecting the output of Metawrap's bin refinement module

This step will produce various outputs and intermediate files in the `4_Bin-refinement` directory:

figures
maxbin2*bins*
*maxbin2*bins.contigs
maxbin2*bins.stats*
*metabat2*bins

metabat2*bins.contigs*
*metabat2*bins.stats
metawrap*50*10*bins*
*metawrap*50*10*bins.contigs
metawrap*50*10*bins.stats*
*work*files

verify that what you have matches the above: `bash ls -l 4_BIN-REFINEMENT/A ls -l 4_BIN-REFINEMENT/B`

`metawrap_50_10_bins` contains the actual bins (MAGs) that were produced by the module:

bin.1.fa
bin.2.fa
bin.3.fa

Verify that you have the same number of bins: `bash ls -l 4_BIN-REFINEMENT/A/metawrap_50_10_bins ls -l 4_BIN-REFINEMENT/B/metawrap_50_10_bins`

You can also check `metawrap_50_10_bins.stats` to see the statistics and taxonomy of the bins:

`bash cat 4_BIN-REFINEMENT/A/metawrap_50_10_bins.stats`

```
bin completeness contamination GC lineage N50 size binner
bin.1 99.57 0.0 0.722 Streptomycetaceae 80779 8364312 binsA
bin.3 99.45 0.0 0.554 Cyanobacteria 369767 2665800 binsA
bin.2 98.82 1.014 0.616 Pseudomonas 117668 5996447 binsAB
```

`bash cat 4_BIN-REFINEMENT/B/metawrap_50_10_bins.stats`

```
bin completeness contamination GC lineage N50 size binner
bin.2 100.0 0.0 0.310 Euryarchaeota 115106 1846511 binsB
bin.1 99.57 0.0 0.722 Streptomycetaceae 80779 8365551 binsA
bin.3 98.82 1.044 0.616 Pseudomonas 119255 6026060 binsA
```

Note: this is a synthetic metagenome constructed from multiple individually sequenced genomes, so the completeness and contamination will be far better than you are likely to generate working on your own data. Do not be concerned if your own results do not look this good.

Now that we consolidated the multiple bin sets from different binners, and gotten an idea of the different taxa / genomes in our samples, we can go ahead and reassemble the reads on a per-bin basis to improve them further.

---

5. Reassembling bins using Metawrap's Reassemble_bins module

The consolidated bin set can be further improved in a lot of cases by reassembling the bins by collecting the reads belonging to each bin, then reassembling them separately. We will use Metawrap's `reassemble_bins` module to accomplish this.

Note: in the event that reassembling the bins does not improve the result, metawrap defaults to the original bin.

Making the Bin_reassembly.sh submission script

We need to create the `Bin_reassembly.sh` script to assemble our two samples. Start by creating and opening this file in the `Scripts` subdirectory: `bash nano Scripts/Bin_reassembly.sh`

You should now be inside an open empty file, which we will gradually add to in order to build up our submission script for reassembling bins.

Adding Slurm scheduler parameters

We need to tell the Slurm scheduler how much resource we want to allocate to the job. The following parameters have been optimised for this dataset. Add them to your open `Bin_reassembly.sh` file by copying and pasting: “‘bash

!/bin/bash

SBATCH --partition=uoa-compute

SBATCH --time=10:00:00

SBATCH --cpus-per-task=16

SBATCH --mem-per-cpu=4G

SBATCH --mail-type=ALL

SBATCH --mail-user=

“`*note: add in your email address after`mail-user=‘ in order to get email updates for the job*

Adding some description of the version the script was written for

It is also good practice to note which version of the package you are using the script was written for, in the event that a newer version becomes available. You can add something like the below which will be useful if you use the script later or send it to somebody else to use. Copy and paste it into your open file: "'bash

This was written for metawrap version 1.3.2

"'

Adding code for loading miniconda and activating the correct environment

Next, we need to make sure that our job is using the environment that we made, so that it has access to Metawrap. Add the below to your open file: "'bash

Source miniconda3 installation and activate environment

source /opt/software/uoa/apps/miniconda3/latest/etc/profile.d/conda.sh conda activate /uoa/scratch/shared/Soil*Microbiology*Group/Training/Metagenomics/envs/Metawrap-v1.3.2

"'

Adding code to set the input and output directories

As above, add the below to your open file: "'bash

Set directory and file name variables

Reads=1*READ-QC Bins=4*BIN-REFINEMENT OutputDir=5_BIN-REASSEMBLY

"' *note: avoid changing the names here, as the rest of this workshop will reference these directories specifically.*

Add code to make the output directories

"'bash

Make output directories

mkdir -p "$OutputDir"/A mkdir -p "$OutputDir"/B

"'

Add code to reassemble the reads into contigs using Metawrap's reassemble_bins module

We want to run the assembly module individually on each sample, so we will do that by adding the following: “‘bash

Run Metawrap's bin reassembly module for sample A

metawrap reassemble*bins -o "$OutputDir"/A -1 "$Reads"/A/final*pure*reads*1.fastq -2 "$Reads"/A/final*pure*reads*2.fastq -t 16 -m 64 -c 50 -x 10 -b "$Bins"/A/metawrap*50*10*bins

Run Metawrap's bin reassembly module for sample B

metawrap reassemble*bins -o "$OutputDir"/B -1 "$Reads"/B/final*pure*reads*1.fastq -2 "$Reads"/B/final*pure*reads*2.fastq -t 16 -m 64 -c 50 -x 10 -b "$Bins"/B/metawrap*50*10*bins

“‘

Here we are using Metawrap's `reassemble_bins` module to reassemble the forward (`-1`) and reverse (`-2`) reads using 16 threads (`-t 16`) and 64GB memory (`-m 64`) based on the refined bins (`-b`). We are also specifying the bin completion (`-c 50`) and contamination (`-x 10`) thresholds the same as for the original binning, and specifying where the output should go (`-o`).

Save the file by using Ctrl+o, hit Enter, then close the file using Ctrl+x.

Running the Bin_reassembly.sh script

You would now be able to run the script using `sbatch Scripts/Bin_reassembly.sh`, but it would take too long to do within the session.

You can find the example results at `/uoa/scratch/shared/Soil_Microbiology_Group/Training/Metagenomi` and link to them in your own directory using:

`bash ln -s /uoa/scratch/shared/Soil_Microbiology_Group/Training/Metagenomics/output/5_BIN-RE 5_BIN-REASSEMBLY`

Inspecting the output of Metawrap's bin_reassembly module

You should now have directory called `5_BIN-REASSEMBLY` that contains the subdirectories `A` and `B`. Within each of these you will have the following:

> original*bins*
> *original*bins.stats
> reassembled*bins*
> *reassembled*bins.checkm
> reassembled*bins.png*

> *reassembled*bins.stats
> reassembly*results.eps*
> *reassembly*results.png
> total.faa
> work_files

Within `reassembled_bins.stats` you will find which version of each bin was used and the associated statistics: `bash cat 5_BIN-REASSEMBLY/A/reassembled_bins.stats`

> bin completeness contamination GC lineage N50 size
> bin.1.orig 99.57 0.0 0.722 Streptomycetaceae 80779 8364312
> bin.2.strict 99.72 0.0 0.554 Cyanobacteria 406760 2668992
> bin.3.orig 98.82 1.014 0.616 Pseudomonas 112980 5996052

`bash cat 5_BIN-REASSEMBLY/B/reassembled_bins.stats`

> bin completeness contamination GC lineage N50 size
> bin.1.permissive 99.91 2.275 0.615 Pseudomonas 190794 6235225
> bin.2.permissive 99.57 0.0 0.722 Streptomycetaceae 90075 8377728
> bin.3.permissive 100.0 0.0 0.310 Euryarchaeota 194754 1850953

You can see that for bin 2 in sample A, and all bins in sample B, reassembly improved the quality of the bins. *Note: the results are not 100% reproducible from run to run, and your output may differ slightly if you run this yourself.*

You can find the final bin files in fasta format (and some other formats), in the `reassembled_bins` subdirectory: `bash ls -l 5_BIN-REASSEMBLY/A/reassembled_bins/*.fa`

> BIN*REASSEMBLY/A/reassembled*bins/bin.1.orig.fa
> BIN*REASSEMBLY/A/reassembled*bins/bin.2.strict.fa
> BIN*REASSEMBLY/A/reassembled*bins/bin.3.orig.fa

`bash ls -l 5_BIN-REASSEMBLY/B/reassembled_bins/*.fa`

> BIN*REASSEMBLY/B/reassembled*bins/bin.1.permissive.fa
> BIN*REASSEMBLY/B/reassembled*bins/bin.2.permissive.fa
> BIN*REASSEMBLY/B/reassembled*bins/bin.3.permissive.fa

These are our MAGs that we can now use for downstream analysis.

---

6. Functionally annotating MAGs using METABOLIC

METABOLIC allows reconstruction of cell metabolism using genomes (including MAGs) as input. This can be done on both a community and individual genome level. Here we will be using METABOLIC in 'community' mode (`METABOLIC-C`), which should allow us to visualise the differences in function between our communities.

However, first we need to change some file suffixes from `.fa` to `.fasta` so that METABOLIC can find our sequence files. We could just do this manually for each file with `mv file.fa file.fasta`, but a script has been provided to do it in a more automated way. Run: `bash bash Scripts/fa_to_fasta.sh`

You will now have the right files for METABOLIC, in a new directory called `MAGs` and we can move onto the next step.

Verify that you have this directory containing the MAGs with: `bash ls -l MAGs/A`

> bin.1.orig.fasta
> bin.2.strict.fasta
> bin.3.orig.fasta

`bash ls -l MAGs/B`

> bin.1.permissive.fasta
> bin.2.permissive.fasta
> bin.3.permissive.fasta

Making the METABOLIC.sh submission script

We need to create the `METABOLIC.sh` script to assemble our two samples. Start by creating and opening this file in the `Scripts` subdirectory: `bash nano Scripts/METABOLIC.sh`

You should now be inside an open empty file, which we will gradually add to in order to build up our submission script for functionally annotating MAGs.

Adding Slurm scheduler parameters

We need to tell the Slurm scheduler how much resource we want to allocate to the job. The following parameters have been optimised for this dataset. Add them to your open `METABOLIC.sh` file by copying and pasting: “‘bash

!/bin/bash

SBATCH --partition=uoa-compute

SBATCH --time=20:00:00

SBATCH --cpus-per-task=40

SBATCH --mem-per-cpu=5G

SBATCH --mail-type=ALL

SBATCH --mail-user=

"`*note: add in your email address after`mail-user=' in order to get email updates for the job*

Adding some description of the version the script was written for

It is also good practice to note which version of the package you are using the script was written for, in the event that a newer version becomes available. You can add something like the below which will be useful if you use the script later or send it to somebody else to use. Copy and paste it into your open file: "'bash

This was written for METABOLIC v4.0

"'

Adding code for loading miniconda and activating the correct environment

Next, we need to make sure that our job is using the environment that we made, so that it has access to METABOLIC. Add the below to your open file: "'bash

Source miniconda3 installation and activate environment

source /opt/software/uoa/apps/miniconda3/latest/etc/profile.d/conda.sh conda activate /uoa/scratch/shared/Soil*Microbiology*Group/Training/Metagenomics/envs/METABOLIC-v4.0

"'

Adding code to set the input and output directories

As above, add the below to your open file: "'bash

Set directory and file name variables

Metabolic=/uoa/scratch/shared/Soil*Microbiology*Group/Training/Metagenomics/METABOLIC GenomesA=MAGs/A GenomesB=MAGs/B OutputDir=6_METABOLIC

“‘ *note: avoid changing the names here, as the rest of this workshop will reference these directories specifically.*

Add code to make the output directories

“‘bash

Make output directories

mkdir -p "$OutputDir"/A mkdir -p "$OutputDir"/B

“‘

Add code to specify paths to metagenomics reads

METABOLIC requires you to specify a file that gives the path to the input reads `-r`, rather than being able to pass those paths into the METABOLIC call directly. To simplify things we have written some short commands that automatically generate these files (which are then deleted once METABOLIC has run). Copy and paste the below into your open `METABOLIC.sh` file: “‘bash

Make file specifying path to metagenomic reads

echo "#Read pairs:" > A.txt echo "1*READ-QC/A/final*pure*reads*1.fastq,1*READ-QC/A/final*pure*reads*2.fastq" » A.txt echo "#Read pairs:" > B.txt echo "1*READ-QC/B/final*pure*reads*1.fastq,1*READ-QC/B/final*pure*reads*2.fastq" » B.txt

“‘

Add code to functionally annotate your genomes and samples using METABOLIC-C

“‘bash

Run METABOLIC-C on genomes for sample A

perl "$Metabolic"/METABOLIC-C.pl -t 40 -m-cutoff 0.75 -in-gn "$GenomesA" -r A.txt -kofam-db full -o "$OutputDir"/A

Run METABOLIC-C on genomes for sample B

perl "$Metabolic"/METABOLIC-C.pl -t 40 -m-cutoff 0.75 -in-gn "$GenomesB" -r B.txt -kofam-db full -o "$OutputDir"/B

“‘

Here we are using METABOLIC in community mode `METABOLIC-C`, with 40 threads (`-t 40`). We are using `m-cutoff 0.75` to use a threshold of 75% of genes in a pathway required to be present before a KEGG module is treated as being present (due to low completion rates of MAGs in most cases, these thresholds cannot be 100% in most cases). We are specifying the path to our MAGs using `-in-gn` , and the path to the raw reads using `-r`. We are using the full rather than small KOfam database (`-kofam-db full`). Finally, we are sending the output to the directory we set earlier in the script, `6_METABOLIC` using `-o`. *Note: there are many more options that you might wish to use / tune for your own data. You can find this using the `--help` flag.*

Finally, clean up those files we had to make specifying the path to the reads

`bash ## Clean up rm A.txt B.txt`

Save the file by using Ctrl+o, hit Enter, then close the file using Ctrl+x.

Running the METABOLIC.sh script

You could now run the script using `sbatch Scripts/METABOLIC.sh` but it is quite resource intensive and not everybody could run it at the same time.

Link to the example results provided using: `bash ln -s /uoa/scratch/shared/Soil_Microbiology_Group/Tr`
`6_METABOLIC`

Inspecting the output of METABOLIC

METABOLIC provides quite a lot of output, but we will look at a subset of that here for simplicity. If you want to learn more about all the outputs it can provide, have a look at the original publication or the Github page. You can use WinSCP or other programs to move the output over to your own machine.

You should have the following files and directories in the `6_METABOLIC/A` and `6_METABOLIC/B` output directories:

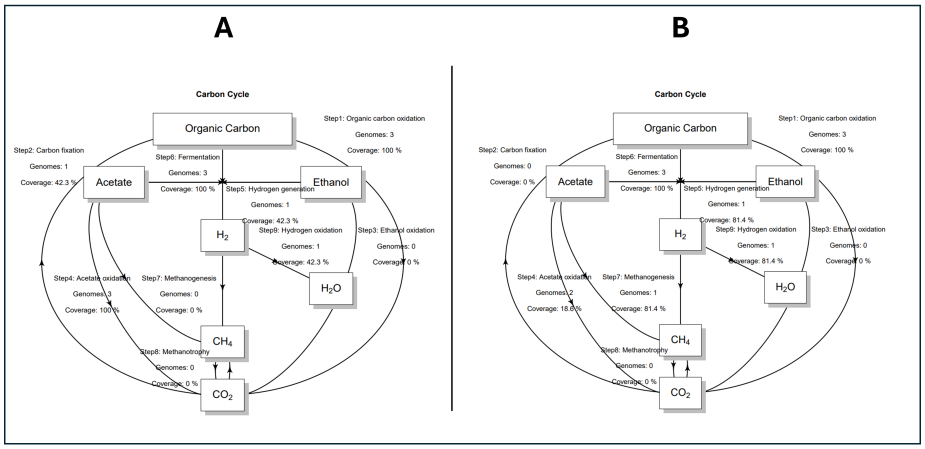
All*gene*collections*mapped.depth.txt*
*Each*HMM*Amino*Acid*Sequence*
*KEGG*identifier*result*
*METABOLIC*Figures
METABOLIC*Figures*Input
METABOLIC*log.log*
*METABOLIC*result.xlsx
METABOLIC*result*each*spreadsheet*
*METABOLIC*run.log
MW-score*result*
*intermediate*files

We will be looking at the following outputs:

METABOLIC*Figures/Nutrient*Cycling*Diagrams/draw*biogeochem*cycles/draw*carbon*cycle*total.pdf
METABOLIC_result.xlsx

To start with, let us have a look at the carbon cycles for each community (sample):

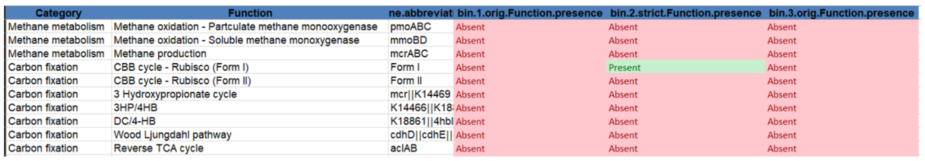
6*METABOLIC/A/METABOLIC*Figures/Nutrient*Cycling*Diagrams/draw*biogeochem*cycles/draw*carbon*cy
6METABOLIC/B/METABOLIC*Figures/Nutrient*Cycling*Diagrams/draw*biogeochem*cycles/draw*carbon*cy*



You can see that in sample A, there is a genome that is predicted to perform carbon fixation, which is not the case in B. This makes sense based on when we inspected the bins at an earlier stage for sample A:

bin	completeness	contamination	GC	lineage	N50	size
bin.1.orig	99.57	0.0	0.722	Streptomycetaceae	80779	8364312
bin.2.strict	99.72	0.0	0.554	Cyanobacteria	406760	2668992
bin.3.orig	98.82	1.014	0.616	Pseudomonas	112980	5996052

You can see we have a cyanobacterium, which are known to be photosynthetic and likely responsible for the carbon fixation here. We can verify this by inspecting `METABOLIC_result.xlsx`. Sheet 2 is called `FunctionHit` and contains various functions and whether they are predicted to be present in each of the MAGs. Inspecting this data for sample A We see the following:



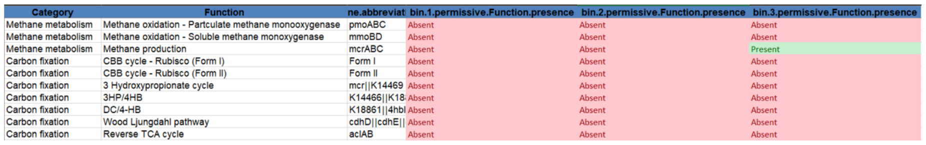
Category	Function	ne.abbreviati	bin.1.orig.Function.presence	bin.2.strict.Function.presence	bin.3.orig.Function.presence
Methane metabolism	Methane oxidation - Partculate methane monooxygenase	pmoABC	Absent	Absent	Absent
Methane metabolism	Methane oxidation - Soluble methane monoxygenase	mmoBD	Absent	Absent	Absent
Methane metabolism	Methane production	mcrABC	Absent	Absent	Absent
Carbon fixation	CBB cycle - Rubisco (Form I)	Form I	Absent	Present	Absent
Carbon fixation	CBB cycle - Rubisco (Form II)	Form II	Absent	Absent	Absent
Carbon fixation	3 Hydroxypropionate cycle	mcr||K14469	Absent	Absent	Absent
Carbon fixation	3HP/4HB	K14466||K18	Absent	Absent	Absent
Carbon fixation	DC/4-HB	K18861||4hbl	Absent	Absent	Absent
Carbon fixation	Wood Ljungdahl pathway	cdhD||cdhE||	Absent	Absent	Absent
Carbon fixation	Reverse TCA cycle	aclAB	Absent	Absent	Absent

We can see that it is indeed the MAG annotated as a cyanobacterium, (bin.2.strict), that is predicted to perform carbon fixation via the CBB (Calvin) cycle - Rubisco (Form I).

We can also see in the carbon cycle diagrams above that, in sample B, there is a genome that is predicted to perform methanogenesis, which is not the case in A. If we inspect the bins for sample B we see:

```
bin completeness contamination GC lineage N50 size
bin.1.permissive 99.91 2.275 0.615 Pseudomonas 190794 6235225
bin.2.permissive 99.57 0.0 0.722 Streptomycetaceae 90075 8377728
bin.3.permissive 100.0 0.0 0.310 Euryarchaeota 194754 1850953
```

Bin 3 is annotated as belonging to the Euryarchaeota, which is a broad kingdom of archaea that include methanogens. If we go on to inspect the `FunctionHit` sheet of `METABOLIC_result.xlsx`, we see the following:



Category	Function	ne.abbreviati	bin.1.permissive.Function.presence	bin.2.permissive.Function.presence	bin.3.permissive.Function.presence
Methane metabolism	Methane oxidation - Partculate methane monooxygenase	pmoABC	Absent	Absent	Absent
Methane metabolism	Methane oxidation - Soluble methane monoxygenase	mmoBD	Absent	Absent	Absent
Methane metabolism	Methane production	mcrABC	Absent	Absent	Present
Carbon fixation	CBB cycle - Rubisco (Form I)	Form I	Absent	Absent	Absent
Carbon fixation	CBB cycle - Rubisco (Form II)	Form II	Absent	Absent	Absent
Carbon fixation	3 Hydroxypropionate cycle	mcr||K14469	Absent	Absent	Absent
Carbon fixation	3HP/4HB	K14466||K18	Absent	Absent	Absent
Carbon fixation	DC/4-HB	K18861||4hbl	Absent	Absent	Absent
Carbon fixation	Wood Ljungdahl pathway	cdhD||cdhE||	Absent	Absent	Absent
Carbon fixation	Reverse TCA cycle	aclAB	Absent	Absent	Absent

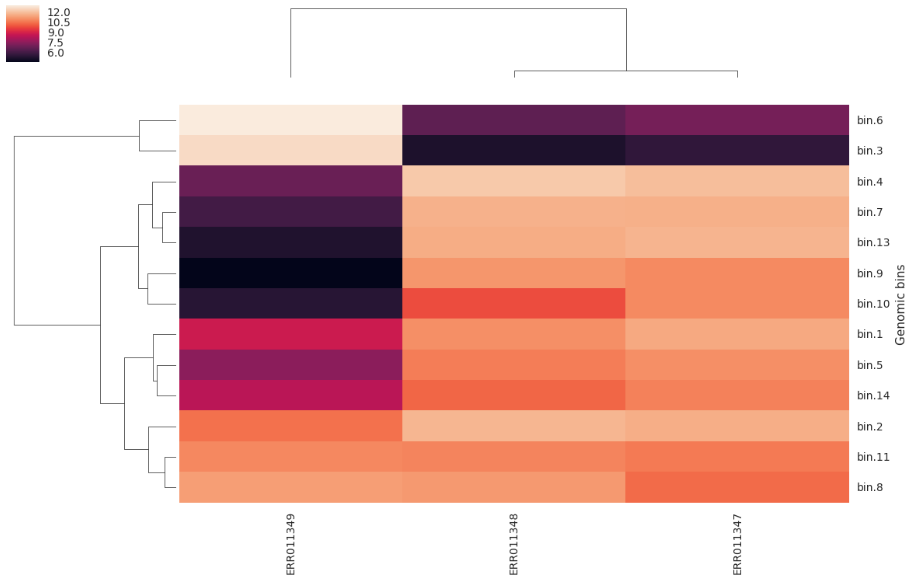
We can see that the MAG annotated as belonging to the Euryarchaeota (bin 3) contains the *mcrABC* operon, which catalyses the final step in methanogenesis.

While this was just a quick look at the output of METABOLIC, there is so much more to delve in to, but that is well beyond the scope of this session. Copy over the output of `6_METABOLIC`, particularly `METABOLIC_result.xlsx`, to your own machine using whichever method you prefer and have a look at all the different outputs it provides; check for your favourite pathways and think about how you might analyse these outputs in your own work.

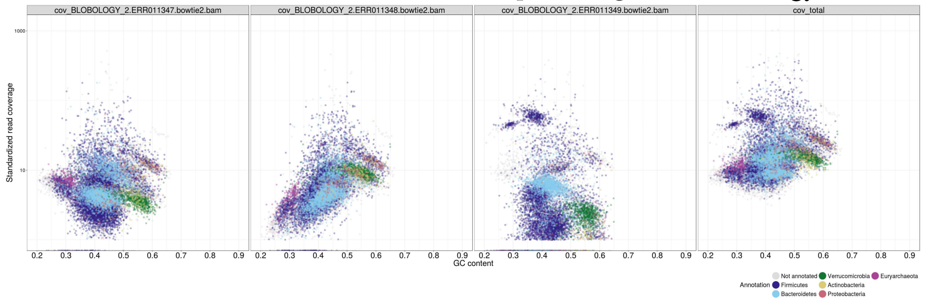
Summary

By this stage, you should be pretty confident in constructing MAGs from raw metagenomic sequences using the scripts you put together in this workshop, and doing some functional annotation. The main things you will need to change are the Slurm scheduler parameters to fit your own data, which will likely be a lot bigger and more complex than what we have used here.

There is also a lot of scope to do far more with `Metawrap` than what we did here. For example, you can use the `Quant_bins` module:



or visualise the communities in each sample using the `blobology` module:



Whatever it is you do, always refer to the documentation for Metawrap and METABOLIC when using them to analyse your data, they are your best guides.

Additional notes

Installing your own instance of miniconda3

If you want to manage your own version of miniconda, so that you can have more control, follow the steps below

Details on how to install miniconda are available here

Below is the quick version pasted from the above link. Make sure to navigate to your home directory first by typing `cd` and hitting Enter, which will take you to your home directory.

Install miniconda: “‘bash mkdir -p ~/miniconda3 wget https://repo.anaconda.com/miniconda/Miniconda3-latest-Linux-x86_64.sh -O ~/miniconda3/miniconda.sh bash ~/miniconda3/miniconda.sh -b -u -p ~/miniconda3 rm -rf ~/miniconda3/miniconda.sh ~/miniconda3/bin/conda init bash

“‘

If this is the first time you have used conda on Maxwell, you will need to **restart your session by logging out and back in.**

Adding channels

You will then need to add some extra channels (repositories to search for packages), in order to be able to create the environments: `bash conda config --add channels conda-forge conda config --add channels bioconda`

It is also recommended to install Mamba, a conda 'drop-in' replacement, before creating the environments to speed things up: `bash conda install -y mamba`

At this point you might normally start creating your environments with the software packages and dependencies that you intend to use. However, Metawrap and METABOLIC are a bit complicated and rely on setting some paths to databases as well as running some setup scripts, which would take too long to do here. A separate Markdown document (@ name.md) is provided for those who wish to do this themselves at a later date.