Tourmaline: a containerized workflow for rapid and iterable amplicon sequence analysis using QIIME 2 and Snakemake

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Abstract

Background: Amplicon sequencing (metabarcoding) is a common method to survey diversity of environmental communities whereby a single genetic locus is amplified and sequenced from the DNA of whole or partial organisms, organismal traces (e.g., skin, mucus, feces), or microbes in an environmental sample. Several software packages exist for analyzing amplicon data, among which QIIME 2 has emerged as a popular option because of its broad functionality, plugin architecture, provenance tracking, and interactive visualizations. However, each new analysis requires the user to keep track of input and output file names, parameters, and commands; this lack of automation and standardization is inefficient and creates barriers to meta-analysis and sharing of results. Findings: We developed Tourmaline, a Python-based workflow that implements QIIME 2 and is built using the Snakemake workflow management system. Starting from a configuration file that defines parameters and input files—a reference database, a sample metadata file, and a manifest or archive of FASTQ sequences—it uses QIIME 2 to run either the DADA2 or Deblur denoising algorithm, assigns taxonomy to the resulting representative sequences, performs analyses of taxonomic, alpha, and beta diversity, and generates an HTML report summarizing and linking to the output files. Features include support for multiple cores, automatic determination of trimming parameters using quality scores, representative sequence filtering (taxonomy, length, abundance, prevalence, or ID), support for multiple taxonomic classification and sequence alignment methods, outlier detection, and automated initialization of a new analysis using previous settings. The workflow runs natively on Linux and macOS or via a Docker container. We ran Tourmaline on a 16S rRNA amplicon dataset from Lake Erie surface water, showing its utility for parameter optimization and the ability to easily view interactive visualizations through the HTML report, QIIME 2 viewer, and R- and Python-based Jupyter notebooks. Conclusions: Automated workflows like Tourmaline enable rapid analysis of environmental and biomedical amplicon data, decreasing the time from data generation to actionable results. Tourmaline is available for download at github.com/aomlomics/tourmaline.
Background

Earth’s environments are teeming with environmental DNA (eDNA): free and cellular genetic material from whole microorganisms (Consortium 2012; Thompson et al. 2017) or remnants of larger macroorganisms (Deiner et al. 2017; Compson et al. 2020). This eDNA can be collected, extracted, and sequenced to reveal the identities and functions of the organisms that produced it. Amplicon sequencing (metabarcoding), whereby a short genomic region is amplified and sequenced using polymerase chain reaction (PCR) from an environmental or experimental community’s eDNA, is a popular method for measuring taxonomic diversity of microorganisms and environmental samples (Zaiko et al. 2015; Deiner et al. 2017; Ruppert, Kline and Rahman 2019). PCR primers have been used to generate amplicons of the bacterial 16S rRNA gene in studies of human and animal-associated microbiota (Turnbaugh et al. 2006; Ahn et al. 2013; Kartzinel et al. 2019), as well as environmental microbiota (Sunagawa et al. 2015; Thompson et al. 2017). Other regions that are commonly targeted include the fungal internal transcribed spacer (ITS) regions between rRNA genes (Abarenkov et al. 2010), the 18S rRNA gene of eukaryotes (Vargas et al. 2015), the mitochondrial cytochrome oxidase I (COI) gene of invertebrate and vertebrate eDNA (Letay et al. 2013), and the mitochondrial 12S rRNA gene of fish (Miya et al. 2015). Information gained from amplicon metabarcoding has far reaching implications for human health (e.g., microbiome research), ecosystem function and conservation, and resource management (Thomsen and Willerslev 2015; Halfvarson et al. 2017a).

Combining datasets from amplicon surveys and performing meta-analysis can reveal patterns impossible to observe from individual studies and also provide more power in statistical analyses. One example of the power of meta-analysis in amplicon surveys is the Earth Microbiome Project (Thompson et al. 2017), which used a single 16S rRNA amplicon method, metadata standard, and denoising algorithm to sequence and analyze over 25,000 microbial communities from around Earth. Standardization of methods is critical for comparability across studies. A lack of standardized methods is one of the main factors limiting cross-comparison of microbiome or eDNA datasets in meta-analyses (Dickie et al. 2018; Harper et al. 2019). While standardizing detailed laboratory methods across many labs in multiple countries is a major challenge, standardizing analysis methods and metadata formats is much more feasible.

A popular approach for standardizing amplicon data analysis is to develop analysis pipelines or workflows (Reiter et al. 2021) to run on local or networked computing resources or in the cloud. Amplicon workflows have become increasingly popular and include pipelines like AnaCapa (Curd et al. 2019), Banzai (https://github.com/jimmyodonnell/banzai), PEMA (Zafeiropoulos et al. 2020), Ampliesq (Straub et al. 2020), Cascabel (Asbun et al. 2019), dadasnake (WeiBecker, Schnabel and Heintz–Buschart 2020), CoMA (Hupfauf et al. 2020), ASAP2 (Tian and Imanian 2022), and tagseq (https://github.com/shuz251/tagseq–qiime2–snakemake). However, stand-alone pipelines typically do not have access to a wide range of datasets, and many pipelines are bespoke workflows with a jumble of custom scripts for the user to navigate. A variation on this model would be a stand-alone workflow that runs on top of a widely used amplicon software tool, such that it could take advantage of the functionality of the underlying tool and evolve with it, while remaining interoperable with any other analyses using this tool. If deployed on the cloud or in a container, it would also be portable enough to run on larger datasets.

QIIME 2 (Bolyen et al. 2019) is a popular software package that provides command-line, Python, and graphical user interfaces for amplicon sequence analysis from raw FASTQ sequences to observation tables, statistical analyses, and interactive visualizations. QIIME 2 supports DADA2 (Callahan et al. 2016) and Deblur (Amir et al. 2017) plugins for denoising amplicon sequence data. Snakemake (Köstler and Rahmann 2012) is a workflow management system that is popular in the bioinformatics community. Snakemake manages input and output files in a defined directory structure, with commands defined in a Snakefile as ‘rules,’ and parameters and initial input files set by the user in a configuration file. Snakemake ensures that only the commands required for requested output files not yet generated are run, saving time and computation when re-running part of a workflow.

Here, we present Tourmaline (github.com/aomlomics/tourmaline), an amplicon analysis pipeline that uses Snakemake to run QIIME 2 commands for core analysis and interactive visualization—plus workflow-specific commands that generate an HTML report of output and summary tables and figures of data and metadata—with rapid analysis aided by workflow iterability and scalability, support for multiple cores, a Docker container, and detailed documentation. After cloning the initial Tourmaline directory from GitHub and setting up the input files and parameters, only a few simple shell commands are required to execute the Tourmaline workflow. Outputs are stored in a standard directory structure that is the same for every Tourmaline run, facilitating data exploration and sharing, parameter optimization, and downstream analysis. Because of this standard directory structure, different runs that utilize different parameters (e.g., DADA2 truncation lengths) can be easily compared, facilitated by a helper script that makes a new copy of the Tourmine directory from an existing one. Every Tourmaline run produces an HTML report containing a summary of metadata and outputs, with links to web-viewable QIIME 2 visualization files. QIIME 2 artifact files can be fed directly into Python- and R-based analysis packages. In addition to running natively on Mac and Linux platforms, Tourmaline can be run in any computing environment using Docker containers. In this paper, we describe the Tourmaline workflow, and apply it to a downsampled 16S rRNA gene dataset from surface waters of Lake Erie. The tutorial includes guidance on evaluating output to refine parameters for the workflow and showcases the HTML report, interactive visualizations, and R- and Python-based analysis notebooks for biological insight into amplicon datasets.

Findings

Workflow

Overview. Tourmaline is a Snakemake-based bioinformatics workflow that operates in a defined directory structure (Fig. 1). Installation involves installing QIIME 2 and other dependencies or installing the Docker container. The starting directory structure is then cloned directly from GitHub and is built out through Snakemake commands, defined as ‘rules’ in Snakefile. Tourmaline provides seven high-level ‘pseudo-rules’ for each of DADA2 paired-end, DADA2 single-end, and Deblur (single-end), running denoising and taxonomic and diversity analy-
Tourmaline is installed by cloning with the command line, QIIME 2, and Snakemake is helpful to generate output directly. The input files and parameters for the workflow are identical for unfiltered and filtered commands, except the outputs go into separate subdirectories. In addition to the 21 pseudo-rules (3 denoising methods with 7 pseudo-rules each), there are 47 regular rules defined in Snakefile that perform the actual QIIME 2, Python, and shell commands of the workflow (Fig. S1).

**Test dataset.** Tourmaline comes with a test dataset of 16S rRNA gene (bacteria/archaea) amplicon data from surface waters of Western Lake Erie in summer 2018 (see Methods). The sequence data were subsampled to 1000 sequences per sample to allow the entire workflow to run in ~10 minutes. This test dataset is used throughout the paper to demonstrate the capabilities of Tourmaline.

**Documentation.** Full instructions for using the Tourmaline workflow, including installation, cloning, and editing the config file, are described in the Tourmaline Wiki at [github.com/aomlomics/tourmaline/wiki](https://github.com/aomlomics/tourmaline/wiki). Some experience with the command line, QIIME 2, and Snakemake is helpful to use Tourmaline; basic tutorials for each of these are provided at [github.com/aomlomics/tutorials](https://github.com/aomlomics/tutorials).

**Installation.** The workflow requires QIIME 2 (version 2021.2) plus several dependencies, which can be installed natively in a Conda environment (instructions on GitHub) or via a Docker container using the Docker image from DockerHub. Tourmaline is installed by cloning the GitHub repository to the current directory with `git clone https://github.com/aomlomics/tourmaline`. This step is repeated any time a new iteration of Tourmaline is needed, and new copies can be initialized using a helper script (described below).

**Snakefile.** As a Snakemake workflow, Tourmaline has as its core files (1) a Snakefile that provides all the commands (rules) that comprise the workflow and (2) a config.yaml file that provides the input files and parameters for the workflow. Snakefile contains all of the commands used by Tourmaline, which invoke QIIME 2 commands, helper scripts (see below), or generate output directly. The main analysis features and options supported by Tourmaline, as specified in Snakefile, are as follows:

- FASTQ sequence import using a manifest file, or use a pre-imported FASTQ.qza file.
- Denoising with DADA2 (Callahan et al. 2016) (paired-end and single-end) and Deblur (Amir et al. 2017) (single-end).
- Feature classification (taxonomic assignment) with options of naïve Bayes (Bokulich et al. 2018), consensus BLAST (Cama-macho et al. 2008), and consensus VSEARCH (Rognes et al. 2016).
- Feature filtering by taxonomy, sequence length, feature ID, and abundance/prevalence.
- De novo multiple sequence alignment with MUSCLE (Edgar 2004), Clustal Omega (Sievers and Higgins 2014), or MAFFT (Katoh and Standley 2013) (with masking) and tree building with FastTree (Price, Dehal and Arkin 2009).
- Outlier detection with odsq (Jehl, Sievers and Higgins 2015).
- Interactive taxonomy barplot.
- Tree visualization using Empress (Cantrell et al. 2021).
- Alpha diversity, alpha rarefaction, and alpha group significance with four metrics: number of observed features, Faith’s phylogenetic diversity, Shannon diversity, and Pielou’s evenness.
- Beta diversity distances, principal coordinates, Emperor (Vázquez-Baeza et al. 2013) plots, and beta group significance (one metadata column) with four metrics: unweighted and weighted UniFrac (Lozupone et al. 2010), Jaccard distance, and Bray–Curtis distance.
- Robust Aitchison PCA and biplot ordination using DEICODE (Martino et al. 2019).

**Config file.** The configuration file config.yaml includes paths to input files and parameters for QIIME 2 commands and other steps. Default settings have been chosen to balance run performance and accuracy and to work with the test data. For user data, all parameters should be checked and possibly adjusted for appropriateness with the dataset; see Table S1, Fig. 1, and the Wiki section Setup for guidance.

**Input files.** Tourmaline requires three categories of input files: (1) Reference database: a FASTA file of reference sequences (refseqs.fna) and a tab-delimited file of taxonomy (ref.tax.tsv) for those sequences, or their imported QIIME 2 artifacts equivalents (refseqs.qza, reftax.qza); (2) Amplicon data: demultiplexed FASTQ sequence files and FASTQ manifest file(s) (manifest_pe.csv, manifest_se.csv) mapping sample names to the location of the sequence files, or their imported QIIME 2 equivalents (fasta_pe.qza, fasta_se.qza); and (3) Metadata: a tab-delimited sample metadata file (metadata.tsv) with sample names in the first column matching those in the FASTQ manifest file. See the Wiki section Setup for guidance on input file paths and use of symbolic links to avoid storing multiple copies of large input files.

**Run the workflow.** The workflow is run using Snakemake commands. For example, if using DADA2 paired-end method without any filtering (see below), the commands would be (1) snakemake dada2_pe_denoise, (2) snakemake dada2_pe_taxonomy_unfiltered, (3) snakemake dada2_pe_diversity_unfiltered, and (4) snakemake dada2_pe_report_unfiltered. Alternatively, the entire workflow can be run at once with the last command, snakemake dada2_pe_report_unfiltered.

**Outputs**

The outputs of each step of Tourmaline are described following a test run with the Lake Erie test data that comes with the GitHub repository. For each command, the main parameters used and list of output files generated in those commands are provided (Fig. 2). Accompanying the list of output files is guidance for evaluating them to choose parameters for subsequent steps (Fig. 2), with screenshots of the Tourmaline-specific output files (Fig. 3) and both QIIME 2 and Tourmaline-specific output files (Fig. S3). A video version of the tutorial is also available on YouTube ([https://youtu.be/1lfyewX5S8X](https://youtu.be/1lfyewX5S8X)).

**Denoise.** The first command is snakemake dada2_pe_denoise (Fig. 2), which imports the FASTQ files and reference database (if not already present in directory or-imported), summarizes the FASTQ data, runs denoising using DADA2, and summarizes the output. In addition to QIIME 2 visualizations of the feature table, representative sequences, and phylogenetic tree, Tourmaline generates a table and scatter plot (repsseqs_properties.tsv, repsseqs_properties_describe.md, repseqs_properties_describe.md).
**Install**

Native installation
- Install Miniconda.
- Install QIIME 2.
- Install Snakemake and dependencies.

OR

Docker container
- Install Docker Desktop.
- Download Docker image.
- Run Docker container.

**Setup**

Clone Tourmaline repository (directory) from GitHub.
- Initialize directory from previous Tourmaline run (optional).
- Edit config.yaml file.
- Link to reference database.
- Organize sequence files and edit fastq manifest file.
- Edit and link to metadata file.

**Run**

Run x_denoise
- $ snakemake -s dada2_pe_denoise

x_taxonomy_unfiltered
x_diversity_unfiltered
x_report_unfiltered
x_taxonomy_filtered
x_diversity_filtered
x_report_filtered

* = sequence of steps
* = output of manual setup
* = output of Snakemake commands
{method} = dada2_pe | dada2_se | deblur_se
{filter} = unfiltered | filtered
qza = QIIME 2 artifact file
qzv = QIIME 2 visualization file

**Input**

`./` (top-level directory)
- Snakefile
- config.yaml
- scripts/

`./00-data/`
- metadata.tsv
- manifest_pe.csv
- repseqs_to_filter_{method}.tsv

**Output**

`./01-imported/`
- refseqs.qza
- reftax.qza
- fastq_pe.qza
- fastq_summary.qzv

`./02-output-{method}-{filter}/`

00-table-repseqs/
- table.qza
- table_summary.qzv
- repseqs.qza
- repseqs.qzv

01-taxonomy/
- taxonomy.qza
- taxonomy.qzv
- taxa_barplot.qzv

02-alignment-tree/
- aligned_repseqs.qza
- root_tree.qza
- root_tree.qzv
- repseqs_properties.tsv
- repseqs_properties.pdf
- repseqs_to_filter_outliers.tsv
- repseqs_to_filter_unassigned.tsv

03-alpha-diversity/
- rarefied_table.qza
- alpha_rarefaction.qzv
- *_vector.qza
- *_group_significance.qzv

04-beta-diversity/
- *_distance_matrix.qza
- *_pcoa_results.qza
- *_emperor.qzv

./03-reports/
- metadata_summary.md
- report_{method}_{/filter}.md
- report_{method}_{/filter}.html

- Example command:

`$ snakemake dada2_pe_denoise`

Figure 1. The Tourmaline workflow. Install natively (macOS, Linux) or using a Docker container. Setup by cloning the Tourmaline repository (directory) from GitHub, initializing the directory from a previous run (optional), editing the configuration file (`config.yaml`, Table S1), creating symbolic links to the reference database files, organizing the sequence files and/or editing the FASTQ manifest file, and editing and creating a symbolic link to the metadata file. Run by calling the Snakemake commands for `denoise`, `taxonomy`, `diversity`, and `report`—or running just the `report` command to generate all output if the parameters do not need to be changed between individual commands. It is recommended but not required to run the `unfiltered` commands before the `filtered` commands. The primary input and output files are listed. Detailed instructions for each step are provided in the Tourmaline Wiki (github.com/aomlomics/tourmaline/wiki).
## Parameters in config.yaml

| Configuration | Value |
|---------------|-------|
| repseq_max_length | 260 |
| repseq_min_length | 0 |
| exclude_terms | unassigned, eukaryota |
| report_theme | github |
| beta_group_pairwise | --p-pairwise |
| beta_group_method | permanova |
| alpha_max_depth | 500 |
| core_sampling_depth | 500 |
| odseq_threshold | 0.025 |
| odseq_bootstrap_replicates | 100 |
| odseq_distance_metric | linear |
| dada2pe_trunc_len_f | 240 |
| dada2pe_trunc_len_r | 190 |
| alignment_method | muscle |
| alignment_muscle_maxiters | 2 |
| alignment_muscle_diags | -diags |
| classify_method | consensus-vsearch |
| dada2pe_trunc_len_r | 190 |
| dada2pe_trunc_len_f | 240 |
| reftax_qza | 01-imported/reftax.qza |
| refseqs_qza | 01-imported/refseqs.qza |
| manifest_pe | 00-data/manifest_pe.csv |

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### Output to evaluate

| Evaluation | Details |
|------------|---------|
| fastq_summary.qzv | - 10 reptags are "Unassigned" and 2 reptags are "d__Eukaryota" -> filter by keywords "unassigned,eukaryota" |
| table_summary.qzv | - Of 16 samples, lowest count per sample is 511, > set core sampling depth (rarefaction) to 500 (check again after filtering) |
| rooted_tree.qzv | - Feature metadata coloring confirms we should filter Unassigned and Eukaryota |
| report_dada2-pe_unfiltered.html | - A summary of the results and metadata and links to output files are presented in this HTML report |

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![Step-by-step tutorial on Tourmaline using the provided test data, which is subsampled from the 16S rRNA amplicon data of a 2018 survey of Western Lake Erie. Key parameters in config.yaml and primary output for each command (pseudo-rule) are listed. Indicated output should be evaluated to determine the appropriate parameters for the next command. Evaluation of the primary outputs and rationale for parameter choice is shown for the test Lake Erie 16S rRNA data that comes with the Tourmaline repository. See Fig. S3 for screenshots of the primary output files.](image-url)
Figure 3. Example of the main outputs of the Tourmaline workflow beyond the QIIME 2 outputs. Contents in panels A, E, F, and G are truncated. Screenshots of additional output files are provided in Fig. S3. See Fig. 2 for commands, parameters, and guidance.
and repseqs_properties.pdf; Fig. 3A–D) of representative se-
quence properties, including sequence length, number of
gaps in the multiple sequence alignment, outlier status, tax-
onomy, and total number of observations in the observa-
tion table. QC can be performed using fastq_summary.qzv
(Fig. S3A) for quality scores and repseqs.qzv (Fig. S3C) or
repseq_lengths.tsv for representative sequence lengths. The
helper script fastq_per_base_sequence_quality_dropoff.py can
be run on the output of FastQC and MultiQC to estimate
and set DADA2 or Deblur truncation lengths (see below)
and then rerun the denoise step. Based on the representative
sequence lengths, filtering by sequence length can also be
set, to be used later in the filtered commands. Choice of
appropriate sampling (rarefaction) depths for the param-
ters ‘alpha_max_depth’ and ‘core_sampling_depth,’ to be
used in the diversity step, can be done by examining ta-
table_summary_features.txt (Fig. 3E), table_summary_samples.txt
(Fig. 3F) and table_summary.qzv (Fig. S3B).

Taxonomy. The second command is snakemake
dada2_pe_taxonomy_unfiltered (Fig. 2), which assigns
taxonomy to the representative sequences using a naive
Bayes classifier or consensus BLAST or VSEARCH method and
generates an interactive taxonomy table and an interactive
barplot of sample taxonomic composition. Choice of taxonomic
groups to be filtered by keyword, to be used later with filtered
commands, can be done by examining taxonomy.qzv (Fig. S3D)
and taxa_barplot.qzv (Fig. S3E).

Diversity. The third command is snakemake
dada2_pe_diversity_unfiltered (Fig. 2), which aligns repre-
sentative sequences using one of three methods, computes
outliers using osseq (Jehl, Sievers and Higgins 2015), and
builds a phylogenetic tree. This step generates lists of represen-
tative sequences that have unassigned taxonomy and were
computed to be outliers, summarizes and plots the
representative sequence properties, performs alpha rarefac-
tion, and runs alpha diversity and beta diversity analyses and
group significance tests using a suite of metrics. Filtering
parameters can be checked by examining rooted_tree.qzv
(Fig. S3F) and repseqs_properties.pdf (Fig. S3G), if desired.
Whether sampling depth was sufficient can be evaluated with
alpha_rarefaction.qzv (Fig. S3J). Alpha and beta diversity pat-
terns and statistically significant differences between groups
can be evaluated with observed_features_group_significance.qzv
(Fig. S3J; other alpha diversity metrics are also provided),
unweighted_unifrac_emperor.qzv (Fig. S3H; other beta diversity
metrics are also provided), and beta_group_significance.qzv
(Fig. S3K).

Report. The fourth and final command is snakemake
dada2_pe_report_unfiltered (Fig. 2), which creates a compre-
hsensive HTML report of parameters, metadata, inputs, out-
puts, and visualizations in a single file. The file report_dada2-
pe_unfiltered.html (Fig. 3G) can be viewed in a web browser,
and the linked output files can be viewed in a browser or down-
loaded and opened with view.qiime2.org (.qzv files) or Mi-
crosoft Excel (.tsv files).

Filtering. After reviewing the unfiltered results—the taxon-
omy summary and taxa barplot, the representative sequence summary plot and table, and the list of unas-
signed and potential outlier representative sequences—the
user may wish to filter (remove) certain representative sequences by taxonomic group or other properties. This
is done by setting the filtering parameters in config.yaml and
providing a list of any individual representative se-
tances to filter, then running the filtered commands of the workflow: snakemake dada2_pe_taxonomy_filtered,

snakemake dada2_pe_diversity_filtered, and snakemake
dada2_pe_report_filtered (Fig. 2). Among the filtered out-
put, the user can check table_summary.qzv (Fig. S3L) to
ensure that the sampling depth after filtering did not ex-
clude samples, and examine rooted_tree.qzv (Fig. S3N) and
repseqs_properties.pdf (Fig. S3O) to check that the desired
representative sequences were filtered. All of the outputs can
be viewed by opening report_dada2_pe_filtered.html (Fig. S3M)
in a web browser.

Downstream analysis & meta-analysis

For users who wish to analyze their output further using
Jupyter notebooks, we provide Python and R notebooks (github.com/aomlomics/tourmaline/tree/master/notebooks)
pre-loaded with popular data analysis and visualization tools for those platforms. These notebooks come ready to
run with Tourmaline output, using relative paths to take advantage of Tourmaline’s defined output file structure. The
notebooks are shown with the tutorial dataset that comes with Tourmaline. We also provide a Python notebook for
meta-analysis, containing commands to merge outputs from multiple Tourmaline runs and then perform diversity analyses
on the merged files.

Python Jupyter notebook. The Python Jupyter notebook
(Fig. S2A) uses the QIIME 2 Visualization and Artifact object
classes, loading Visualization and Artifact objects from the .qzv
and.qza Tourmaline output files. Before running the notebook,
the denoising method, filtering mode, and alpha and beta di-
versity metrics to be used can be specified by changing vari-
able assignments at the beginning of the notebook. The note-
book renders Visualization objects for the feature table sum-
mary, representative sequences summary, phylogetic tree,
taxonomy, taxa bar plot, alpha diversity group significance,
and beta diversity principal coordinates analysis (PCoA) Em-
peror plot. Artifact objects can be viewed as a Pandas (McKin-
ney 2010) DataFrame or Series. The notebook generates Pan-
das DataFrames for the feature table, taxonomy, reference se-
quency properties, and metadata, and a Pandas Series for alpha
diversity. Static plots are generated from some of these tables
using Seaborn (Qalieh et al. 2017).

R Jupyter notebook. The R Jupyter notebook (Fig. S2B) im-
ports Tourmaline artifact (.qza) files using qiime2R (Bisanz
2018) and uses common R packages for analyzing and visualiz-
ing amplicon sequencing data, including phyloseq (Halfvarson
et al. 2017b), tidyverse (Wickham et al. 2019), and vegan (Oks-
sanen et al. 2020). The notebook covers how to import QIIME
2 count and taxonomy artifact files from Tourmaline into an R
environment, merge and manipulate the resulting data frames
into a single phyloseq object, and estimate and plot diversity
metrics and taxonomy bar plots of the 16S community using
phyloseq and other packages. As with the Python notebook,
a set of variables can be specified at the beginning of the R
notebook to define specific denoising, filtering, and diversity
metrics. After reading in the metadata file and merging to a phyloseq object, we define plotting parameters that can be eas-
ily modified by the user to customize the R visualizations.

Meta-analysis notebook. The meta-analysis notebook
(Fig. S2C) guides the user through running Tourmaline on two
separate datasets, merging the outputs (feature tables, represen-
tative sequences, and taxonomies) and metadata, and per-
forming some basic diversity analyses on the merged output.
For simplicity, the two datasets are derived from the test data
that comes with Tourmaline. The commands provided could be
applied to any set of Tourmaline outputs that the user wishes
to combine in a meta-analysis. The only requirement is that
the sequenced region must be the same across the datasets for
the results to make sense. This notebook is a simple ex-
ample that demonstrates Tourmaline’s capacity to facilitate merg-
ing of outputs and meta-analysis. Many additional analyses
are possible on the merged output, such as demonstrated in published microbiome meta-analyses (Thompson et al. 2017; Delgado–Baquerizo et al. 2018).

Helper scripts & parameter optimization

Tourmaline comes with several helper scripts that are run automatically with the workflow or run directly by the user. See the Wiki section Setup for more information.

**Initialize a new Tourmaline directory.** From the main directory of a newly cloned Tourmaline directory, the script `initialize_dir_from_existing_tourmaline_dir.sh` will copy `config.yaml` and `Snakefile` from an existing tourmaline directory, remove the test files, then copy the data files and symbols from the existing Tourmaline directory. This is useful when performing a new analysis on the same dataset. The user can clone a new copy of Tourmaline, run this script to copy everything from the old copy to the new one, then make desired changes to the parameters.

**Create a FASTQ manifest file.** Two scripts help create the manifest file that points Tourmaline to the FASTQ sequence files. (1) `create_manifest_from_fastq_directory.py` creates a FASTQ manifest file from a directory of FASTQ files. (2) `match_manifest_to_metadatap.py` takes an existing FASTQ manifest file and generates two new manifest files (paired-end and single-end) corresponding to the samples in the provided metadata file.

**Determine optimal truncation length.** If FastQC and MultiQC have been run for Read 1 and Read 2, `fastqc_per_base_sequence_quality_dropoff.py` will determine the position where median per-base sequence quality drops below some fraction (default: 0.90) of its maximum value. This is useful for defining 3’ truncation positions in DADA2 and Deblur (`'dada2pe_trunc_len_f','dada2se_trunc_len',' and deblur_trunc_length.py').

**Parameter optimization.** The helper scripts and Tourmaline’s standard directory structure enable testing and comparison of different parameter sets to optimize a workflow. By making multiple copies of the directory and populating settings with `initialize_dir_from_existing_tourmaline_dir.sh` script, varying one or a small number of parameters, and running the workflow multiple times in parallel, outputs can be compared visually or programmatically to see the effects of parameter choices and choose a final set. To illustrate this, we analyzed the full dataset of the 2018 Lake Erie 16S rRNA study (BioProject PRJNA679730). Running `fastqc_per_base_sequence_quality_dropoff.py` had suggested that a forward truncation length of 240 bp and reverse truncation length of 190 bp would strike a balance between sequence length and quality, but we wanted to test a full range of truncation lengths. We tested the effects of varying the forward and reverse truncation lengths from 100 bp to 250 bp in 50-bp increments on the distribution of representative sequence length (Fig. S4A) and the number of reads assigned to Eukaryota (Fig. S4B), a group potentially amplified by these primers but with longer representative sequences. This analysis helped choose a set of truncation lengths that would capture a large diversity of target organisms.

**Parallelization & benchmarks**

A typical amplicon sequencing dataset is much larger than the test dataset that comes with the Tourmaline repository and will take considerably longer to process. To evaluate runtimes with a real-world dataset, we ran Tourmaline on the full dataset of the 2018 Lake Erie 16S rRNA study (BioProject PRJNA679730), which is the dataset from which the test dataset was subsampled. This dataset was sequenced with 2x300–bp Illumina MiSeq sequencing and consists of 96 samples having an average of 120,338 paired reads per sample, for a total of 11,552,448 paired reads. Processing was performed using the Tourmaline Docker container running on a 2017 iMac Pro with an 18-core 2.3-GHz Intel Xeon W processor and 64 GB RAM (32 GB RAM allocated for the Docker container). Speed improvements with parallelization were tested by running Snakemake with either 1 or 8 cores (parameter: `--cores`). Each main step in the workflow (denoise, taxonomy, diversity, and report; unfiltered commands) was run and timed separately. Times would be expected to be similar for filtered commands except that the denoise rule does not need to be rerun. The results (Table 1) show that a relatively large dataset of ~100 samples with ~100,000 sequences per sample can be processed with a single core in ~5 hours. Dramatic speed improvements are possible with multiple cores, with this same dataset being processed in ~2 hours when 8 cores were used.

**Biological insights**

The purpose of performing amplicon sequencing or metabarcoding is to reveal patterns of diversity, community structure, and biological (or environmental) drivers within diverse ecosystems. Whether the system of study is microbial communities in an environmental or biomedical setting or trace environmental DNA in an aquatic or terrestrial system, the kinds of biological questions being asked are similar. Tourmaline supports biological insight in two important ways: (1) by supporting the most popular analysis tools and packages in use today, with capacity to expand as new tools are developed; (2) by providing multiple ways to view the output, giving everyone from experts to novices a platform to visualize and query the output.

Through its core QIIME 2 functionality and downstream support for R and Python data science packages, Tourmaline enables analysis of the core metrics of microbial and eDNA diversity: taxonomic composition, within-sample diversity (alpha diversity), and between-sample diversity (beta diversity). Examining our analysis of the tutorial dataset (Fig. S3), we can see how Tourmaline facilitates insight into Western Lake Erie microbial communities. The interactive barplot (Fig. S3E) provides rapid insights: the most abundant bacterial families in the 5.0–µm fraction are Sporichthyaceae and SAR11 Clade III; the most abundant bacterial family in the 0.22–µm fraction is Cyanobacteria (the toxic cyanobacterial family Microcystaceae is less abundant), with the largest component assigned as chloroplasts, which can be filtered in a subsequent run; at the domain level, a small fraction of unassigned and Eukaryota-assigned sequences are observed, which can also be filtered. The alpha diversity results show that the 5.0–µm fraction has greater within-sample diversity (number of observed features) than the 0.22–µm fraction (Fig. S3J) and that this diversity appears to be saturated, with a relatively small sampling depth of ~350 sequences per sample sufficient to observe these values (Fig. S3J). However, because a large fraction of the 0.22–µm sequences were identified as chloroplast, filtering out those sequences in a future run would be warranted and provide more accurate diversity results. The beta diversity results show that 16S communities are distinguished both by location (Open Water vs. Western Boundary) and size fraction (0.22–µm vs. 5.0–µm) (Fig. S3H). From this simple tutorial dataset, we demonstrate the use of Tourmaline to analyze environmental amplicon data, in this case revealing the importance of pore size when filtering water samples for microbial sequencing and the presence of spatial variability (regardless of pore size) among microbial communities in Lake Erie.

The ability to view Tourmaline output files with multiple
interfaces provides access to researchers with different back-
grounds. For users experienced with the Unix command line, the
diverse output file types, organized in a defined directory
structure, can be queried and analyzed using a wide array of
data science tools; anything that can be done with QIIME 2
output and other common sequence diversity output files
types can be done with Tourmaline output. For data scientists most
comfortable with Jupyter notebooks, the prebuilt Python and
R notebooks come ready to work with Tourmaline output and
rapidly enable biological discovery from amplicon data. For ca-
Tual users, the web-based report and QIIME 2 visualizations
provide a user-friendly onramp to view and interact with the
output and other common sequence diversity output files.

Table 1. Benchmarking and parallel processing results from running the full 2018 Lake Erie 16S rRNA dataset through Tourmaline with either 1 or 8 cores using a Tourmaline Docker container allocated with 32 GB RAM running on an 18-core iMac Pro (2017). The Snakemake command used the parameter --cores 1 or --cores 8, and parameters in config.yaml specifying the number of threads for individual rules were set to 1 or 8, respectively. Times reported are the elapsed real time between invocation and termination and are reported as HH:MM:SS. Times do not include the initial step of importing FASTQ files into a QIIME 2 archive (fastq_pe.qza), which took ~2 minutes. Parameters shown in the last column are those most relevant to the runtimes. Unless otherwise noted, the parameters used were the defaults in config.yaml.

| Rule                          | Time (-- cores 1) | Time (--cores 8) | Parameters & details |
|------------------------------|------------------|------------------|----------------------|
| dada2_pe_denoise             | 02:05:43         | 00:38:20         | method: dada2-pe     |
|                              |                  |                  | 96 samples * 120,338 sequences per sample = 11,552,448 total sequences |
| dada2_pe_taxonomy_unfiltered | 01:31:55         | 00:32:39         | classify_method: consensus-vsearch |
|                              |                  |                  | 12,379 representative sequences |
| dada2_pe_diversity_unfiltered| 01:18:09         | 01:13:49         | alignment_method: muscle |
|                              |                  |                  | alignment_muscle_maxiters: 2 |
|                              |                  |                  | alignment_muscle_diags: -diags |
|                              |                  |                  | odseq_distance_metric: linear |
|                              |                  |                  | odseq_bootstrap_replicates: 100 |
|                              |                  |                  | odseq_threshold: 0.025 |
|                              |                  |                  | 12,379 representative sequences |
|                              |                  |                  | (lengths: min 240, max 418, avg 258) |
| dada2_pe_report_unfiltered   | 00:00:05         | 00:00:05         | –                     |
| Total                        | 04:55:52         | 02:04:43         | –                     |

Conclusions

Tourmaline provides a comprehensive platform for amplicon
sequence analysis that enables rapid and iterable processing
and inference of microbiome and eDNA metabarcoding data. It
has multiple features that enhance usability and interoperabil-
ity:

- **Portability.** Native support for Linux and macOS in addition to Docker containers, enabling it to run on desktop, cluster, and cloud computing platforms.
- **QIIME 2.** The core commands of Tourmaline, including the DADA2 and Deblur packages, are all commands of QIIME 2, one of the most popular amplicon sequence analysis software tools available. Users can print all of the QIIME 2 and other shell commands of a workflow before or while run-
ning the workflow.
- **Snakemake.** Managing the workflow with Snakemake pro-
vides several benefits:
  - **Configuration file** contains all parameters in one file, so the user can see what the workflow is doing and make changes for a subsequent run.
  - **Directory structure** is the same for every Tourmaline run, so the user always knows where outputs are.
  - **On-demand commands** mean that only the commands required for output files not yet generated are run, sav-
ing time and computation when re-running part of a workflow.
- **Parameter optimization.** The configuration file and stan-
dard directory structure make it simple to test and compare different parameter sets to optimize a workflow.
- **Visualizations and reports.** Every Tourmaline run produces an HTML report containing a summary of metadata and outputs, with links to web-viewable QIIME 2 visualization files.
- **Downstream analysis.** Analyze the output of single or mul-
tiple Tourmaline runs programmatically, with qiime2r in R or the QIIME 2 Artifact API in Python, using the provided R and Python notebooks or other code.

Through its streamlined workflow and broad functional-
ity, Tourmaline enables rapid response and biological discov-
ery in any system where amplicon sequencing is applied, from
biomedical and environmental microbiology to eDNA for fis-
sheries and protected or invasive species. The QIIME 2–based
interactive visualizations it generates allow users to quickly
compare differences between samples and groups of samples
in their taxonomic composition, within–sample diversity (al-
Sample collection and DNA extraction

Water samples were collected using a long-range autonomous underwater vehicle (LRAUV, Monterey Bay Aquarium Research Institute) equipped with a third-generation environmental sample processor (3G-ESP, Monterey Bay Aquarium Research Institute) (Fargett et al. 2015). For each sample, water was filtered through stacked 5.0-µm (top) and 0.22-µm (bottom) Durapore filters (EMD Millipore) held in custom 3G-ESP ‘archive’ cartridges and preserved in-cartridge with RNAlater (Thermo Fisher). DNA extraction was performed using the Qiagen DNeasy Blood and Tissue kit.

Amplicon sequencing

Extracted DNA was amplified using a BioScientific NEXTFlex 16S V4 Amplicon–Seq Kit 2.0 (NOVA-520999/Custom NOVA-4203-04) (BioScientific, Austin, TX, USA). Target-specific regions of the forward and reverse primers in the 16S V4 Amplicon–Seq kit were custom ordered to follow the Earth Microbiome Project 16S Illumina Amplicon Protocol: forward primer 515F 5’-GTGTCACGGCAGCGGTAA-3’ (Parada, Needham and Fuhrman 2016) and reverse primer 806R 5’-GGACTACNVGGGTWTCTAAT-3’ (Apprill et al. 2015). 16S rRNA amplicons were pooled and sequenced on an Illumina MiSeq with 2 x 300-bp chemistry at the University of Michigan Advanced Genomics Core. Demultiplexed sequences were deposited in NCBI under BioProject PRJNA679738.

Availability of supporting source code

• Tourmaline is available by cloning the GitHub repository at https://github.com/aomlomics/tourmaline. Tourmaline is released under a 3-clause BSD license.
• Full installation and usage instructions are available from the Tourmaline Wiki at https://github.com/aomlomics/tourmaline/wiki.
• A Docker image is available from DockerHub at https://hub.docker.com/r/aomlomics/tourmaline. This docker image is based on a QIIME 2 Docker image hosted at https://github.com/qiime2/qiime2/blob/master/LICENSE, and the QIIME 2 license can be found at https://github.com/qiime2/qiime2/blob/master/LICENSE.
• Additional code and data from the full 2018 Lake Erie dataset is available at https://github.com/aomlomics/erie.

Availability of supporting data

• The test 16S dataset (1000 sequences per sample) is available directly from the GitHub repository at https://github.com/aomlomics/tourmaline.
• Reference databases are available for 16S rRNA at https://docs.qiime2.org/2021.2/data-resources/#silva-16s-rrna and for 18S–ITS rRNA at https://unite.ut.ee/repository.php.
• Output for the tutorial using the included test data are available from Zenodo at https://doi.org/10.5281/zenodo.5044532.
• A snapshot of the GitHub repository will be available from Zenodo upon publication.

Abbreviations

ASV: amplicon sequence variant; COI: cytochrome oxidase I; DAG: directed acyclic graph; eDNA: environmental DNA; ITS: internal transcribed spacer; NMDS: non-metric dimensional scaling; PCoA: principal coordinates analysis; PCR: polymerase chain reaction; QIIME: Quantitative Insights Into Microbial Ecology.

Competing interests

The authors declare that they have no competing interests.

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Author contributions

The Tourmaline workflow was designed and developed by L.R. Thompson. Code was tested by L.R. Thompson, N.V. Patin, S.R. Anderson, and S.J. Lim. The Docker image was built by N.V. Patin and L.R. Thompson. Data analysis and visualization of the case study were done by S.R. Anderson. Analysis notebooks were developed by L.R. Thompson, S.R. Anderson, and G. Sanderson. Samples were collected by P.A. Den Uyl and K.D. Goodwin. DNA was extracted and prepared for sequencing by P.A. Den Uyl. The manuscript was written by L.R. Thompson,
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Table S1. Parameters in the configuration file, config.yaml, that the user may edit as necessary. Additional parameters not shown may also be edited. The default configuration file is provided in the top level of the GitHub repository. The file format of config.yaml, YAML (yet another markup language), is a simple markup language that is used by Snakemake to specify parameters for a workflow.

| Parameter                                | Description                                                                 | Recommendation                                                                 | Help                                      |
|------------------------------------------|-----------------------------------------------------------------------------|-------------------------------------------------------------------------------|-------------------------------------------|
| dada2pe_trunc_len_f                      | Truncate bases (integer) from the 3’ (right) ends of reads in DADA2.        | Choose values that maximize length but remove low-quality ends. Note that DADA2 paired-end mode requires a minimum overlap of 12 bp to merge Read 1 and Read 2. See the section below “Sequence quality control and choice of truncation length” for instructions on using the included script fastqc_per_base_sequence_quality_dropoff.py. | dada2 denoise-paired; dada2 denoise-single |
| dada2pe_trunc_len_r                      | Truncate bases (integer) from the 5’ (left) ends of reads in DADA2.         | Depending on your amplicon sequencing method, and if trimming was not done prior to running Tourmaline, you may have primer sequences, indexes, and/or adapters on the 5’ ends of your reads. If so, set this parameter to remove those bases. If not, set this parameter to zero. Note that 5’ trimming (this parameter) is done after 3’ truncation (above parameter). | dada2 denoise-paired; dada2 denoise-single |
| dada2se_trunc_len                        | Truncate bases (integer) from the 3’ (right) ends of reads in Deblur.       | Choose values that maximize length but remove low-quality ends. See the section below “Sequence quality control and choice of truncation length” for instructions on using the included script fastqc_per_base_sequence_quality_dropoff.py. | deblur denoise-other                      |
| dada2pe_pooling_method                   | DADA2 pooling method.                                                       | Choose pseudo for pseudo-pooling or independent for no pooling.              | dada2 denoise-paired; dada2 denoise-single |
| dada2se_pooling_method                   | DADA2 chimera method.                                                       | Choose pooled if pseudo-pooling otherwise consensus or none.                | dada2 denoise-paired; dada2 denoise-single |
| dada2se_chimera_method                   | Multiple sequence alignment method.                                         | Choose muscle or clustalo for best accuracy or mafft for faster results.    | muscle; clustalo; mafft                   |
| dada2pe_chimera_method                   | Taxonomic classification method.                                            | Choose naive-bayes for best accuracy or consensus-blast for faster results. | feature-classifier                       |
| deblur_trim_length                       | Filter terms (taxa) from taxonomy.                                          | Specify terms (comma-separated, no spaces) to find in taxonomy and filter out (case-insensitive), or provide a nonsense term to skip this step when filtering. | taxa filter-seq                          |
| odseq_bootstrap_replicates               | Set minimum and maximum sequence lengths to filter representative sequences by. | Limits are inclusive, i.e., sequences will be retained if greater than or equal to minimum, less than or equal to maximum. Leave defaults (0, 10000) to do no filtering. | taxa filter-seq                          |
| odseq_min_abundance                      | Set minimum abundance and prevalence limits to filter representative sequences by. | Limit is inclusive, i.e., sequences will be retained if greater than or equal to minimum. Leave default (0) to do no filtering. | taxa filter-seq                          |
| odseq_distance_metric                    | Distance metric for odseq.                                                  | Choose metric from: linear, affine.                                         | odseq                                    |
| odseq_threshold                          | Probability to be at the right of the bootstrap scores distribution when computing outliers. Tune this parameter depending on the diversity and occurrence of outliers in the MSA. | Choose more replicates for more robust detection of outliers, fewer replicates for faster processing. | odseq                                    |
| odseq_max_depth                          | Rarefaction depth (integer) for core diversity metrics.                    | Choose a value that balances sequencing depth (more is better) with number of samples retained (more is better). | diversity core-metrics-phylogenetic       |
| odseq_min_depth                          | Rarefaction depth (integer) for alpha rarefaction.                         | Choose a value that balances sequencing depth (more is better) with number of samples retained (more is better). | diversity alpha-rarefaction              |
| odseq_min_prevalence                     | Rarefaction depth (integer) for odseq.                                      | Choose a category that may differentiate your samples. This analysis can be rerun with different columns by renaming the output file and changing the value in config.yaml before running again. | diversity beta-group-significance        |
| odseq_max_prevalence                     | Rarefaction depth (integer) for odseq.                                      | Choose from: github, gothic, newsprint, night, pixyll, whitey.              | Typora theme gallery                     |
Figure S1. Directed acyclic graphs (DAGs) of the Tourmaline workflow for the DADA2 paired-end method from start to report with (a) unfiltered commands and (b) filtered commands. This figure was generated from the test data that comes with the repository by running the commands (a) `snakemake dada2_pe_report_unfiltered --dag | dot -Tpdf -Grankdir=LR -Gnodesep=0.1 -Granksep=0.1 > dag_pe_report_unfiltered.pdf` and (b) `snakemake dada2_pe_report_filtered --dag | dot -Tpdf -Grankdir=LR -Gnodesep=0.1 -Granksep=0.1 > dag_pe_report_filtered.pdf`. For a simpler graph, substitute `--rulegraph` for `--dag` in the above commands.
Figure S2. Screenshots of Tourmaline’s included Python and R Jupyter notebooks running the provided test data. Both notebooks are designed to run out-of-the-box with the Tourmaline output from any dataset. (A) The Tourmaline Python notebook loads and displays sample metadata, feature metadata (representative sequences properties and taxonomy), static plots generated by Seaborn, and interactive QIIME 2 visualizations. (B) The Tourmaline R notebook demonstrates how to load .qza files (counts and taxonomy) into R, merge files with metadata into a single phyloseq object, and generate high-quality visualizations of community diversity and taxonomy using phyloseq and suite of tidyverse packages (e.g., ggplot2). (C) The Tourmaline meta-analysis notebook walks through the merging of two sets of Tourmaline outputs and performing some basic diversity analyses on the merged files. The number of processed datasets being merged in the meta-analysis can be increased by adding additional inputs to the commands.
files (.qzv, .pdf, .html) are useful both for data evaluation and discovery and for biological insight.
Figure S4. Effect of truncation length parameters on (A) the distribution of representative sequence length and (B) the number of reads assigned to Eukaryota in the full 2018 Lake Erie 16S rRNA amplicon data.