RESEARCH ARTICLE

Metagenomics workflow for hybrid assembly, differential coverage binning, metatranscriptomics and pathway analysis (MUFFIN)

Renaud Van Damme[1,2]*, **Martin Hölzer**[3], **Adrian Viehweger**[3,4], **Bettina Müller**[1], **Erik Bongcam-Rudloff**[2], **Christian Brandt**[2,5]

1 Department of Molecular Sciences, Swedish University of Agricultural Sciences, Uppsala, Sweden, **2** Department Animal Breeding and Genetics, Bioinformatics section, Swedish University of Agricultural Sciences, Uppsala, Sweden, **3** RNA Bioinformatics and High-Throughput Analysis, Friedrich Schiller University Jena, Jena, Germany, **4** Department of Medical Microbiology, University Hospital Leipzig, Leipzig Germany, **5** Institute for Infectious Diseases and Infection Control, Jena University Hospital, Jena, Germany

* renaud.van.damme@slu.se

Citation: Van Damme R, Hölzer M, Viehweger A, Müller B, Bongcam-Rudloff E, Brandt C (2021) Metagenomics workflow for hybrid assembly, differential coverage binning, metatranscriptomics and pathway analysis (MUFFIN). PLoS Comput Biol 17(2): e1008716. https://doi.org/10.1371/journal.pcbi.1008716

Editor: Mihaela Pertea, Johns Hopkins University, UNITED STATES

Received: July 20, 2020

Accepted: January 17, 2021

Published: February 9, 2021

Peer Review History: PLOS recognizes the benefits of transparency in the peer review process; therefore, we enable the publication of all of the content of peer review and author responses alongside final, published articles. The editorial history of this article is available here: https://doi.org/10.1371/journal.pcbi.1008716

Data Availability Statement: All subset files for testing the pipeline are available from https://osf.io/m5czv/ MUFFIN is available at https://github.com/

Abstract

Metagenomics has redefined many areas of microbiology. However, metagenome-assembled genomes (MAGs) are often fragmented, primarily when sequencing was performed with short reads. Recent long-read sequencing technologies promise to improve genome reconstruction. However, the integration of two different sequencing modalities makes downstream analyses complex. We, therefore, developed MUFFIN, a complete metagenomic workflow that uses short and long reads to produce high-quality bins and their annotations. The workflow is written by using Nextflow, a workflow orchestration software, to achieve high reproducibility and fast and straightforward use. This workflow also produces the taxonomic classification and KEGG pathways of the bins and can be further used for quantification and annotation by providing RNA-Seq data (optionally). We tested the workflow using twenty biogas reactor samples and assessed the capacity of MUFFIN to process and output relevant files needed to analyze the microbial community and their function. MUFFIN produces functional pathway predictions and, if provided *de novo* metatranscript annotations across the metagenomic sample and for each bin. MUFFIN is available on github under GNUv3 licence: https://github.com/RVanDamme/MUFFIN.

Author summary

Determining the entire DNA of environmental samples (sequencing) is a fundamental approach to gain deep insights into complex bacterial communities and their functions. However, this approach produces enormous amounts of data, which makes analysis time intense and complicated. We developed the Software "MUFFIN," which effortlessly untangle the complex sequencing data to reconstruct individual bacterial species and determine their functions. Our software is performing multiple complicated steps in

RVanDamme/MUFFIN under GNU General Public License version 3.

Funding: This study was funded by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) – BR 5692/1-1 and BR 5692/1-2. This material is based upon work supported by Google Cloud. BM was funded by FORMAS, grant number 942-2015-1008. MH is supported by the Collaborative Research Centre AquaDiva (CRC 1076 AquaDiva) of the Friedrich Schiller University Jena, funded by the DFG. MH appreciates the support of the Joachim Herz Foundation by the add-on fellowship for interdisciplinary life science. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

parallel, automatically allowing everyone with only basic informatics skills to analyze complex microbial communities.

For this, we combine two sequencing technologies: "long-sequences" (nanopore, better reconstruction) and "short-sequences" (Illumina, higher accuracy). After the reconstruction, we group the fragments that belong together ("binning") via multiple approaches and refinement steps while also utilizing the information from other bacterial communities ("differential binning"). This process creates hundreds of "bins" whereas each represents a different bacterial species with a unique function. We automatically determine their species, assess each genome's completeness, and attribute their biological functions and activity ("transcriptomics and pathways"). Our Software is entirely freely available to everyone and runs on a good computer, compute cluster, or via cloud.

This is a *PLOS Computational Biology* Software paper.

Introduction

Metagenomics is widely used to analyze the composition, structure, and dynamics of microbial communities, as it provides deep insights into uncultivatable organisms and their relationship to each other [1–5]. In this context, whole metagenome sequencing is mainly performed using short-read sequencing technologies, predominantly provided by Illumina. Not surprisingly, the vast majority of tools and workflows for the analysis of metagenomic samples are designed around short reads. However, long-read sequencing technologies, as provided by PacBio or Oxford Nanopore Technologies (ONT), retrieve genomes from metagenomic datasets with higher completeness and less contamination [6]. The long-read information bridges gaps in a short-read-only assembly that often occur due to intra- and interspecies repeats [6]. Complete viral genomes can be already identified from environmental samples without any assembly step via nanopore-based sequencing [7]. Combined with a reduction in cost per gigabase [8] and an increase in data output, the technologies for sequencing long reads quickly became suitable for metagenomic analysis [9–12]. In particular, with the MinION, ONT offers mobile and cost-effective sequencing device for long reads that paves the way for the real-time analysis of metagenomic samples. Currently, the combination of both worlds (long reads and high-precision short reads) allows the reconstruction of more complete and more accurate metagenome-assembled genomes (MAGs) [6].

One of the main challenges and bottlenecks of current metagenome sequencing studies is the orchestration of various computational tools into stable and reproducible workflows to analyze the data. A recent study from 2019 involving 24,490 bioinformatics software resources showed that 26% of all these resources are not currently online accessible [13]. Among 99 randomly selected tools, 49% were deemed 'difficult to install,' and 28% ultimately failed the installation procedure. For a large-scale metagenomics study, various tools are needed to analyze the data comprehensively. Thus, already during the installation procedure, various issues arise related to missing system libraries, conflicting dependencies and environments, or operating system incompatibilities. Even more complicating, metagenomic workflows are computing intense and need to be compatible with high-performance compute clusters (HPCs), and thus different workload managers such as SLURM or LSF. We combined the workflow manager Nextflow [14] with virtualization software (so-called 'containers') to generate reproducible results in various working environments and allow full parallelization of the workload to a higher degree.

Several workflows for metagenomic analyses have been published, including MetaWRAP (v1.2.1) [15], Anvi'o [16], SAMSA2 [17], Humann [18], MG-Rast [19], ATLAS [20], or Sunbeam [21]. Unlike those, MUFFIN allows for a hybrid metagenomic approach combining the strengths of short and long reads. It ensures reproducibility through the use of a workflow manager and reliance on either install-recipes (Conda [22]) or containers (Docker [23], Singularity).

Design and implementation

MUFFIN integrates state-of-the-art bioinformatic tools via Conda recipes or Docker/Singularity containers for the processing of metagenomic sequences in a Nextflow workflow environment (Fig 1). MUFFIN executes three steps subsequently or separately if intermediate results, such as MAGs, are available. As a result, a more flexible workflow execution is possible. The three steps represent common metagenomic analysis tasks and are summarized in Fig 1:

1. Assemble: Hybrid assembly and binning
2. Classify: Bin quality control and taxonomic assessment
3. Annotate: Bin annotation and KEGG pathway summary

The workflow takes paired-end Illumina reads (short reads) and nanopore-based reads (long reads) as input for the assembly and binning and allows for additional user-provided read sets for differential coverage binning. Differential coverage binning facilitates genome bins with higher completeness than other currently used methods [24]. Step 2 will be executed automatically after the assembly and binning procedure or can be executed independently by providing MUFFIN a directory containing MAGs in FASTA format. In step 3, paired-end RNA-Seq data can be optionally supplemented to improve the annotation of bins.

On completion, MUFFIN provides various outputs such as the MAGs, KEGG pathways, and bin quality/annotations. Additionally, all mandatory databases are automatically downloaded and stored in the working directory or can be alternatively provided via an input flag.

Step 1—Assemble: Hybrid assembly and binning. The first step (**Assembly and binning**) uses metagenomic nanopore-based long reads and Illumina paired-end short reads to obtain high-quality and highly complete bins. The short-read quality control is operated using fastp (v0.20.0) [25]. Optionally, Filtlong (v0.2.0) [26] can be used to discard long reads below a length of 1000 bp. The hybrid assembly can be performed according to two principles, which differ substantially in the read set to begin with. The default approach starts from a short-read assembly where contigs are bridged via the long reads using metaSPAdes (v3.13.2) [27–29]. Alternatively, MUFFIN can be executed starting from a long-read-only assembly using metaFlye (v2.8) [30,31] followed by polishing the assembly with the long reads using Racon (v1.4.13) [32] and medaka (v1.0.3) [33] and finalizing the error correction by incorporating the short reads using multiple rounds of Pilon (v1.23) [34]. Both approaches should be chosen based on the available amount of raw read data available to users. E.g., if more short read data is available, meta-spades should be the choice (long reads are "supplemental"). If more long-read data is available, e.g.,> 15 Gigabases (corresponds to a full MinION or GridION flow cell) [35] flye should be used as the assembly approach.

Binning is one of the most crucial steps during metagenomic analysis besides assembly. Therefore, MUFFIN combines three different binning software tools, respectively CONCOCT (v1.1.0) [36], MaxBin2 (v2.2.7) [37], and MetaBAT2 (v2.13) [38] and refine the obtained bins via MetaWRAP (v1.3) [15]. The user can provide additional read data sets (short or long

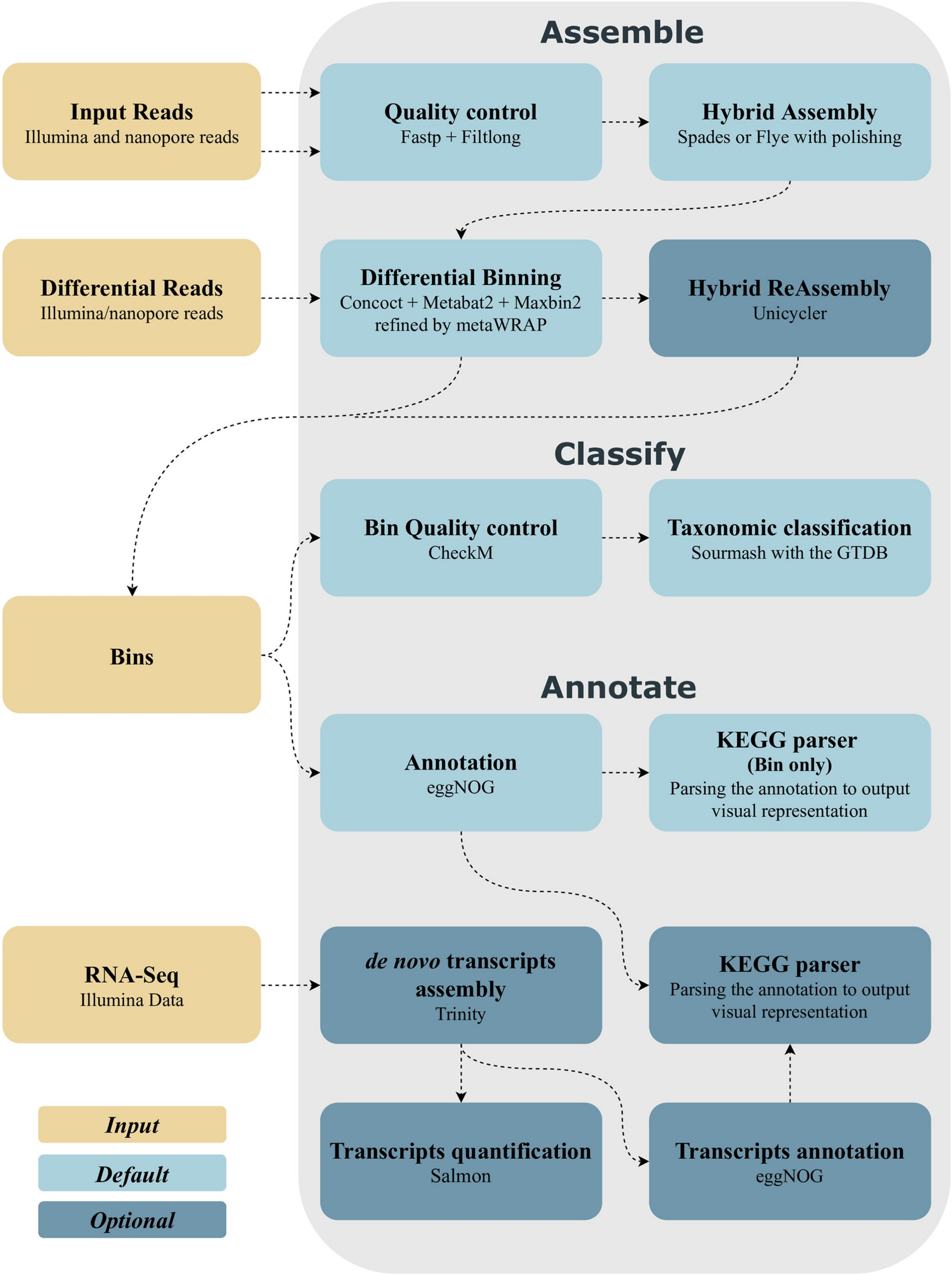

Assemble
Input Reads
Illumina and nanopore reads
Quality control
Fastp + Filtlong
Hybrid Assembly
Spades or Flye with polishing
Differential Reads
Illumina/nanopore reads
Differential Binning
Concoct + Metabat2 + Maxbin2
refined by metaWRAP
Hybrid ReAssembly
Unicycler
Classify
Bin Quality control
CheckM
Taxonomic classification
Sourmash with the GTDB
Bins
Annotate
Annotation
eggNOG
KEGG parser
(Bin only)
Parsing the annotation to output visual representation
RNA-Seq
Illumina Data
de novo transcripts assembly
Trinity
KEGG parser
Parsing the annotation to output visual representation
Transcripts quantification
Salmon
Transcripts annotation
eggNOG
Input
Default
Optional

Fig 1. Simplified overview of the MUFFIN workflow. All three steps (Assemble, Classify, Annotate) from top to bottom are shown. The RNA-Seq data for Step 3 (Annotate) is optional. Differential reads are other read data sets that are solely used for "differential coverage binning" to improve the overall binning performance.

https://doi.org/10.1371/journal.pcbi.1008716.g001

reads) to perform automatically differential coverage binning to assign contigs to their bins better.

Moreover, an additional reassembly of bins has shown the capacity to increase the completeness and N50 while decreasing the contamination of some bins [15]. Therefore, MUFFIN allows for an optional reassembly to improve the continuity of the MAGs further. This reassembly is performed by retrieving the reads belonging to one bin and doing an assembly with Unicycler (v0.4.7) [39]. As each reassembly might improve or worsen each bin, this process is optional and therefore deactivated by default. Individual manual curation is necessary by the user to compare each bin before and after reassembly, as described by Uritskiy *et al.* [15].

To support a transparent and reproducible metagenomics workflow, all reads that cannot be mapped back to the existing high-quality bins (after the refinement) are available as an output for further analysis. These "unused" reads could be further analyzed by other tools such as Kraken2 [40], Kaiju [41], or centrifuge [42] for read classification, "What the Phage" [43] to search for phages, mi-faser [44] for functional annotation of the reads or even use these reads as a new input to run MUFFIN.

Step 2—Classify: Bin quality control and taxonomic assessment. In the second step (**Bin quality control and taxonomic assessment**), the quality of the bins is evaluated with CheckM (v1.1.3) [45] followed by assigning a taxonomic classification to the bins using sourmash (v2.0.1) [46] and the Genome Taxonomy Database (GTDB release r89) [47]. The GTDB was chosen as it contains many unculturable bacteria and archaea–this allows for monophyletic species assignments, which other databases do not assure [35,48]. Moreover, the coherent taxonomic classifications and more accurate taxonomic boundaries (e.g., for class, genus, etc.) proposed by GTDB substantially increases the general classification accuracy [48]. The user can also analyze other bin sets in this step regardless of their origin by providing a directory with multiple FASTA files (bins).

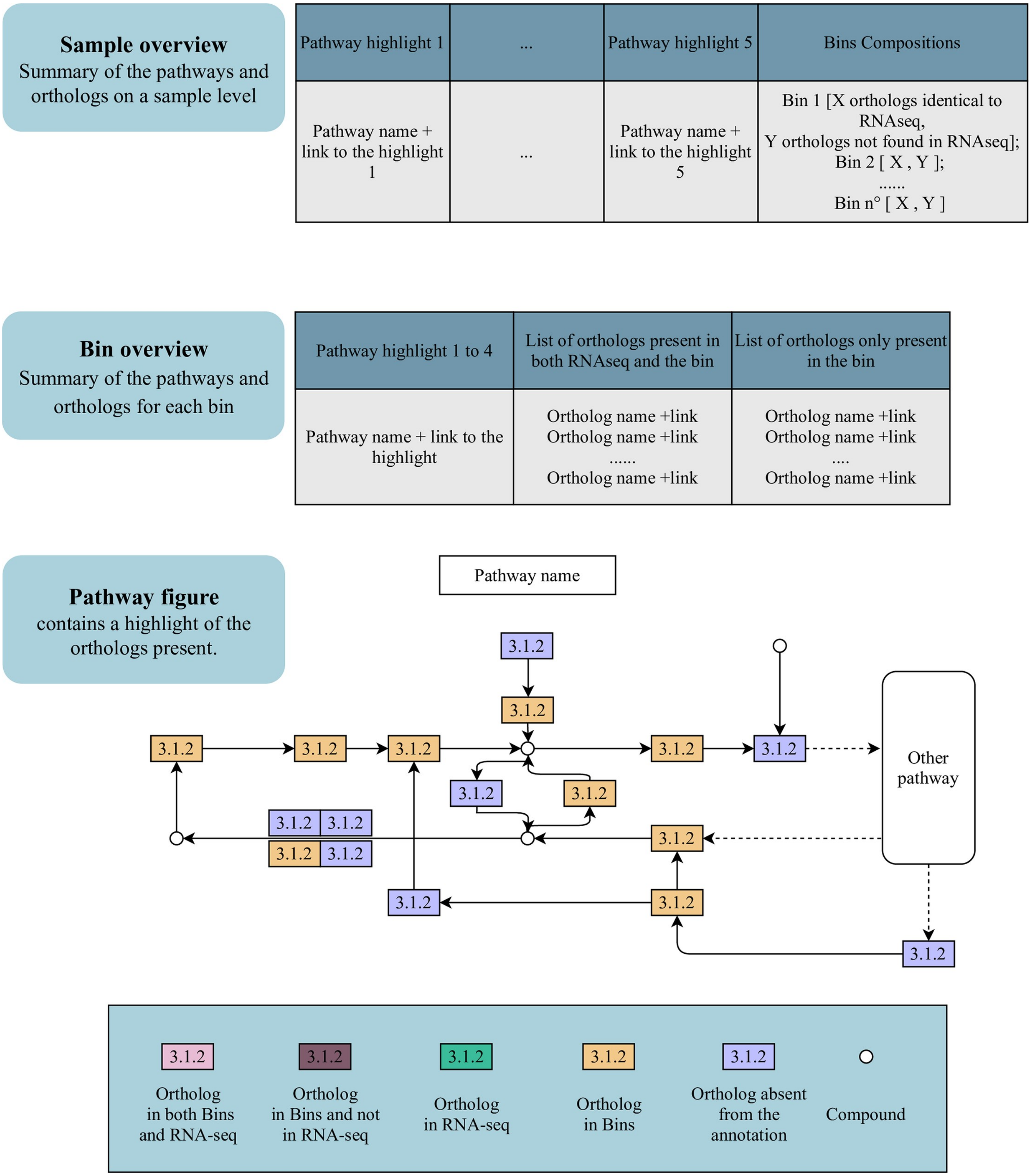
Step 3—Annotate: Bin annotation and KEGG pathway summary. The last step of MUFFIN (**Bin annotation and output summary**) comprises the annotation of the bins using eggNOG-mapper (v2.0.1) [49] and the eggNOG database (v5) [50]. If RNA-Seq data of the metagenome sample is provided (Illumina, paired-end), quality control using fastp (v0.20.0) [25] and a *de novo* metatranscript assembly using Trinity (v2.9.1) [51] followed by quantification of the metatranscripts by mapping of the RNA-seq reads using Salmon (v1.0) [52] are performed. Lastly, the metatranscripts are annotated using eggNOG-mapper (v2.0.1) [49]. Again, the annotation by eggnog-mapper provides a wide array of annotation information such as the GO terms, the NOG terms, the BiGG reaction, CAZy, KEGG orthology, and pathways.

These gene annotations are parsed and visualized in KEGG pathways for each sample and bin. The expression of low and high abundant genes present in the bins is shown. If only bin sets are provided without any RNA-Seq data, the pathways of all the bins are created based on gene presence alone. The KEGG pathway results are summarized in detail as interactive HTML files (example snippet: Fig 2).

Like step 2, this step can be directly performed with a bin set created via another workflow.

Running MUFFIN and version control

MUFFIN (V1.0.3, 10.5281/zenodo.4296623) requires only two dependencies, which allows an easy and user-friendly workflow execution. One of them is the workflow management system



Sample overview
Summary of the pathways and orthologs on a sample level

Pathway highlight 1	...	Pathway highlight 5	Bins Compositions
Pathway name + link to the highlight 1	...	Pathway name + link to the highlight 5	Bin 1 [X orthologs identical to RNAseq, Y orthologs not found in RNAseq]; Bin 2 [X , Y]; ...... Bin n° [X , Y]

Bin overview
Summary of the pathways and orthologs for each bin

Pathway highlight 1 to 4	List of orthologs present in both RNAseq and the bin	List of orthologs only present in the bin
Pathway name + link to the highlight	Ortholog name +link Ortholog name +link ...... Ortholog name +link	Ortholog name +link Ortholog name +link .... Ortholog name +link

Pathway figure
contains a highlight of the orthologs present.

Pathway name
3.1.2
Other pathway
3.1.2 Ortholog in both Bins and RNA-seq
3.1.2 Ortholog in Bins and not in RNA-seq
3.1.2 Ortholog in RNA-seq
3.1.2 Ortholog in Bins
3.1.2 Ortholog absent from the annotation
○ Compound

Fig 2. Example snippets of the sub-workflow results of step 3 (Annotate).

https://doi.org/10.1371/journal.pcbi.1008716.g002

Nextflow [14] (version 20.07+), and the other can be either Conda ^{20}[22] as a package manager or Docker [23] / Singularity to use containerized tools. A detailed installation process is available on https://github.com/RVanDamme/MUFFIN. Each MUFFIN release specifies the Nextflow version it was tested on, but any version of MUFFIN V1.0.2+ will work with nextflow version 20.07+. A Nextflow-specific version can always be directly downloaded as an executable file from https://github.com/nextflow-io/nextflow/releases, which can then be paired with a compatible MUFFIN version via the -r flag.

Results

We chose Nextflow for the development of our metagenomic workflow because of its direct cloud computing support (Amazon AWS, Google Life Science, Kubernetes), various ready-to-use batch schedulers (SGE, SLURM, LSF), state-of-the-art container support (Docker, Singularity), and accessibility of a widely used software package manager (Conda). Moreover, Nextflow [14] provides a practical and straightforward intermediary file handling with process-specific work directories and the possibility to resume failed executions where the work ceased. Additionally, the workflow code itself is separated from the 'profile' code (which contains Docker, Conda, or cluster related code), which allows for a convenient and fast workflow adaptation to different computing clusters without touching or changing the actual workflow code.

The entire MUFFIN workflow was executed on 20 samples from the Bioproject PRJEB34573 (available at ENA or NCBI) using the Cloud Life Sciences API (google cloud) with docker containers. This metagenomic bioreactor study provides paired-end Illumina and nanopore-based data for each sample [35]. We used five different Illumina read sets of the same project for differential coverage binning, and the workflow runtime was less than two days for all samples. MUFFIN was able to retrieve 1122 MAGs with genome completeness of at least 70% and contamination of less than 10% (Fig 3). In total, MUFFIN retrieved 654 MAGs with genome completeness of over 90%, of which 456 have less than 2% contamination out of the 20 datasets. For comparison, a recent study was using 134 publicly available datasets from different biogas reactors and retrieved 1,635 metagenome-assembled genomes with genome completeness of over 50% [53].

Exemplarily, we investigated the impact of additional reassembly of each bin for five samples (Fig 3). The N50 was increased by an average of 6–7 fold across all samples. Twenty-six bins of the five samples had an N50 ranging between 1 to 3 Mbases. Reassembly of bins has shown the capacity to increase the completeness and N50 while decreasing the contamination of some bins [15]. This is in line with our samples as some bins benefit more from this step than others. In general, while we observed a general increase in N50 for most bins, the genome quality based on checkM metrics (completeness, contamination) was slightly increasing or decreasing for individual bins.

Discussion

The analysis of metagenomic sequencing data evolved as an emerging and promising research field to retrieve, characterize, and analyze organisms that are difficult to cultivate. There are numerous tools available for individual metagenomics analysis tasks, but they are mainly developed independently and are often difficult to install and run. The MUFFIN workflow gathers the different steps of a metagenomics analysis in an easy-to-install, highly reproducible, and scalable workflow using Nextflow, which makes them easily accessible to researchers.

MUFFIN utilizes the advantages of both sequencing technologies. Short-reads provide a better representation of low abundant species due to their higher coverage based on read count. Long-reads are utilized to resolve repeats for better genome continuity. This aspect is

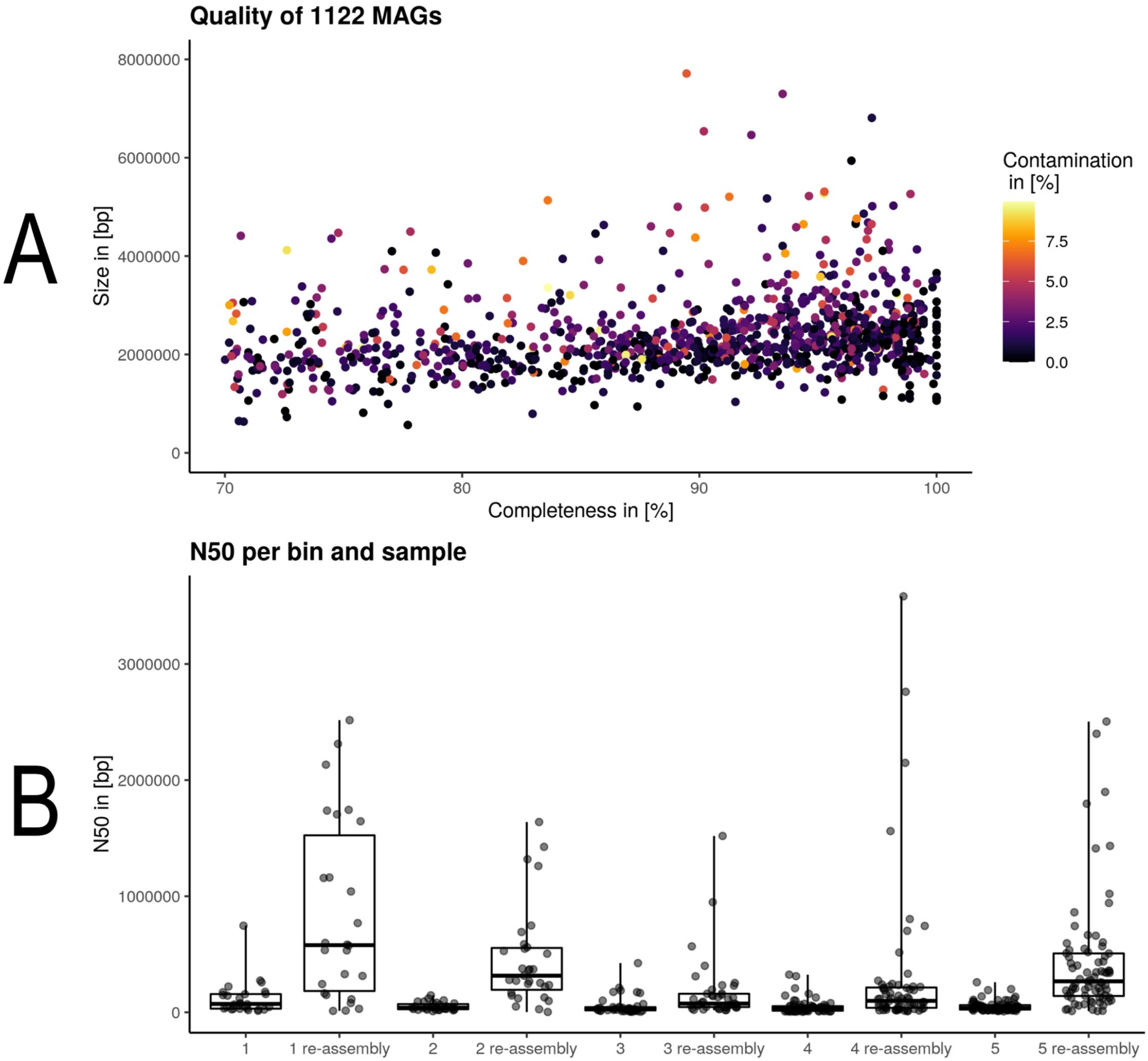


Fig 3. Quality of meta-assembled genomes (MAGs). [A] Quality overview of 1122 MAGs by plotting size to completeness and coloring based on contamination level. [B] N50 comparison between each bin of five selected samples from the Bioproject PRJEB34573 before and after individual bin reassembly.

https://doi.org/10.1371/journal.pcbi.1008716.g003

further utilized via the final reassembly step after binning, which is an optional step due to the additional computational burden which solely aims to improve genome continuity.

Another critical aspect is the full support of differential binning, for both long and short reads, via a single input option. The additional coverage information from other read sets of similar habitats allows for the generation of more concise bins with higher completeness and less contamination because more coverage information is available for each binning tool to decide which bin each contig belongs to.

With supplied RNA-Seq data, MUFFIN is capable of enhancing the pathway results present in the metagenomic sample by incorporating this data as well as the general expression level of the genes. Such information is essential to further analyze metagenomic data sets in-depth, for example, to define the origin of a sample or to improve environmental parameters for production reactors such as biogas reactors. Knowing whether an organism expresses a gene is a crucial element in deciding whether more detailed analysis of that organism in the biotope where the sample was taken is necessary or not.

MUFFIN utilizes a large number of tools to provide a comprehensive analysis of metagenomics samples. The associated tools were mainly chosen based on benchmark performance, e.g., assembly [29,31,54–56], polishing [55], binning [15], annotation for pathways [49], taxonomic classification [47], however stability and workflow compatibility was also an important factor to consider. Due to the modular coding structure of nextflow DSL2 language, MUFFIN can quickly adapt towards better tools or improved versions if necessary, in the future.

MUFFIN executes a de novo assembly of the RNA-seq reads instead of a mapping of the reads against the MAGs to avoid bias and error during the mapping. Indeed, not all the DNA reads were assembled or binned and present in the last step (annotation). Thus we might miss transcripts on the sample level. In addition, for similar genes, it's impossible to know to which organism the reads should map to. By using metatranscripts and comparing the annotations of the metatranscripts to the annotation of the MAGs, we avoid those issues.

Availability and future directions

MUFFIN is an ongoing workflow project that gets further improved and adjusted. The modular workflow setup of MUFFIN using Nextflow allows for fast adjustments as soon as future developments in hybrid metagenomics arise, including the pre-configuration for other workload managers. MUFFIN can directly benefit from the addition of new bioinformatics software such as for differential expression analysis and short-read assembly that can be easily plugged into the modular system of the workflow. Another improvement is the creation of an advanced user and wizard user configuration file, allowing experienced users to tweak the different parameters of the different software as desired.

MUFFIN will further benefit from different improvements, in particular by graphically comparing the generated MAGs via a phylogenetic tree. Furthermore, a convenient approach to include negative controls is under development to allow the reliable analysis of super-low abundant organisms in metagenomic samples.

MUFFIN is publicly available at https://github.com/RVanDamme/MUFFIN under the GNU general public license v3.0. Detailed information about the program versions used and additional information can be found in the GitHub repository. All tools used by MUFFIN are listed in the S1 Table. The Docker images used in MUFFIN are prebuilt and publicly available at https://hub.docker.com/u/nanozoo, and the GTDB formatted for sourmash (v2.0.1)[46] usage is publicly available at https://osf.io/m5czv/. The MAGs produced by the 20 samples; the template of the output of MUFFIN (README_output.txt); the subset data use in the test profile of MUFFIN (subset_data.tar.gz); and the results of MUFFIN on the subset data with and without RNA using both flye and spades are also available at https://osf.io/m5czv/. The Version of MUFFIN presented in this paper is (V1.0.3, 10.5281/zenodo.4296623).

Supporting information

S1 Table. List of the MUFFIN task, the softwares and versions.
(XLSX)

Acknowledgments

We want to thank Hadrien Gourlé and Moritz Buck for the valuable insights into metagenomic analysis and annotation.

Author Contributions

Conceptualization: Renaud Van Damme, Christian Brandt.

Data curation: Renaud Van Damme, Bettina Müller.

Formal analysis: Renaud Van Damme, Christian Brandt.

Funding acquisition: Christian Brandt.

Investigation: Renaud Van Damme.

Methodology: Renaud Van Damme, Martin Hölzer, Adrian Viehweger, Christian Brandt.

Project administration: Christian Brandt.

Resources: Bettina Müller, Erik Bongcam-Rudloff.

Software: Renaud Van Damme, Martin Hölzer, Adrian Viehweger, Erik Bongcam-Rudloff, Christian Brandt.

Supervision: Christian Brandt.

Validation: Renaud Van Damme, Bettina Müller.

Visualization: Renaud Van Damme.

Writing – original draft: Renaud Van Damme.

Writing – review & editing: Martin Hölzer, Adrian Viehweger, Erik Bongcam-Rudloff, Christian Brandt.

References

1. Handelsman J, Rondon MR, Brady SF, Clardy J, Goodman RM. Molecular biological access to the chemistry of unknown soil microbes: a new frontier for natural products. Chem Biol. 1998; 5: R245–R249. https://doi.org/10.1016/s1074-5521(98)90108-9 PMID: 9818143
2. De R. Metagenomics: aid to combat antimicrobial resistance in diarrhea. Gut Pathog. 2019; 11: 47. https://doi.org/10.1186/s13099-019-0331-8 PMID: 31636714
3. Mukherjee A, Reddy MS. Metatranscriptomics: an approach for retrieving novel eukaryotic genes from polluted and related environments. 3 Biotech. 2020; 10: 71. https://doi.org/10.1007/s13205-020-2057-1 PMID: 32030340
4. Grossart H-P, Massana R, McMahon KD, Walsh DA. Linking metagenomics to aquatic microbial ecology and biogeochemical cycles. Limnol Oceanogr. 2020; 65: S2–S20. https://doi.org/10.1002/lno.11382
5. Carabeo-Pérez A, Guerra-Rivera G, Ramos-Leal M, Jiménez-Hernández J. Metagenomic approaches: effective tools for monitoring the structure and functionality of microbiomes in anaerobic digestion systems. Appl Microbiol Biotechnol. 2019; 103: 9379–9390. https://doi.org/10.1007/s00253-019-10052-5 PMID: 31420693
6. Overholt WA, Hölzer M, Geesink P, Diezel C, Marz M, Küsel K. Inclusion of Oxford Nanopore long reads improves all microbial and viral metagenome-assembled genomes from a complex aquifer system. Environ Microbiol. 2020; 22: 4000–4013. https://doi.org/10.1111/1462-2920.15186 PMID: 32761733
7. Assembly-free single-molecule nanopore sequencing recovers complete virus genomes from natural microbial communities | bioRxiv. [cited 3 Dec 2020]. Available: https://www.biorxiv.org/content/10.1101/619684v1 PMID: 32075851
8. Wetterstrand KA. DNA Sequencing Costs: Data. In: www.genome.gov/sequencingcostsdata [Internet]. 5 Feb 2020 [cited 5 Feb 2020]. Available: www.genome.gov/sequencingcostsdata

9. Somerville V, Lutz S, Schmid M, Frei D, Moser A, Irmler S, et al. Long-read based de novo assembly of low-complexity metagenome samples results in finished genomes and reveals insights into strain diversity and an active phage system. BMC Microbiol. 2019; 19: 143. https://doi.org/10.1186/s12866-019-1500-0 PMID: 31238873

10. Warwick-Dugdale J, Solonenko N, Moore K, Chittick L, Gregory AC, Allen MJ, et al. Long-read viral metagenomics captures abundant and microdiverse viral populations and their niche-defining genomic islands. PeerJ. 2019;7. https://doi.org/10.7717/peerj.6800 PMID: 31086738

11. Driscoll CB, Otten TG, Brown NM, Dreher TW. Towards long-read metagenomics: complete assembly of three novel genomes from bacteria dependent on a diazotrophic cyanobacterium in a freshwater lake co-culture. Stand Genomic Sci. 2017;12. https://doi.org/10.1186/s40793-017-0232-8 PMID: 28138356

12. Suzuki Y, Nishijima S, Furuta Y, Yoshimura J, Suda W, Oshima K, et al. Long-read metagenomic exploration of extrachromosomal mobile genetic elements in the human gut. Microbiome. 2019; 7: 119. https://doi.org/10.1186/s40168-019-0737-z PMID: 31455406

13. Mangul S, Martin LS, Eskin E, Blekhman R. Improving the usability and archival stability of bioinformatics software. Genome Biol. 2019; 20: 47. https://doi.org/10.1186/s13059-019-1649-8 PMID: 30813962

14. Di Tommaso P, Chatzou M, Floden EW, Barja PP, Palumbo E, Notredame C. Nextflow enables reproducible computational workflows. Nat Biotechnol. 2017; 35: 316–319. https://doi.org/10.1038/nbt.3820 PMID: 28398311

15. Uritskiy GV, DiRuggiero J, Taylor J. MetaWRAP—a flexible pipeline for genome-resolved metagenomic data analysis. Microbiome. 2018; 6: 158. https://doi.org/10.1186/s40168-018-0541-1 PMID: 30219103

16. Eren AM, Esen ÖC, Quince C, Vineis JH, Morrison HG, Sogin ML, et al. Anvi'o: an advanced analysis and visualization platform for 'omics data. PeerJ. 2015; 3: e1319. https://doi.org/10.7717/peerj.1319 PMID: 26500826

17. Westreich ST, Treiber ML, Mills DA, Korf I, Lemay DG. SAMSA2: a standalone metatranscriptome analysis pipeline. BMC Bioinformatics. 2018; 19: 175. https://doi.org/10.1186/s12859-018-2189-z PMID: 29783945

18. Abubucker S, Segata N, Goll J, Schubert AM, Izard J, Cantarel BL, et al. Metabolic Reconstruction for Metagenomic Data and Its Application to the Human Microbiome. PLOS Comput Biol. 2012; 8: e1002358. https://doi.org/10.1371/journal.pcbi.1002358 PMID: 22719234

19. Meyer F, Paarmann D, D'Souza M, Olson R, Glass E, Kubal M, et al. The metagenomics RAST server–a public resource for the automatic phylogenetic and functional analysis of metagenomes. BMC Bioinformatics. 2008; 9: 386. https://doi.org/10.1186/1471-2105-9-386 PMID: 18803844

20. Kieser S, Brown J, Zdobnov EM, Trajkovski M, McCue LA. ATLAS: a Snakemake workflow for assembly, annotation, and genomic binning of metagenome sequence data. BMC Bioinformatics. 2020; 21: 257. https://doi.org/10.1186/s12859-020-03585-4 PMID: 32571209

21. Clarke EL, Taylor LJ, Zhao C, Connell A, Lee J-J, Fett B, et al. Sunbeam: an extensible pipeline for analyzing metagenomic sequencing experiments. Microbiome. 2019; 7: 46. https://doi.org/10.1186/s40168-019-0658-x PMID: 30902113

22. Anaconda Software distribution. Anaconda | The World's Most Popular Data Science Platform. In: https://anaconda.com [Internet]. 5 Feb 2020 [cited 5 Feb 2020]. Available: https://www.anaconda.com/

23. Boettiger C. An introduction to Docker for reproducible research. ACM SIGOPS Oper Syst Rev. 2015; 49: 71–79. https://doi.org/10.1145/2723872.2723882

24. Albertsen M, Philip H, Skarshewski A, Nielsen K, Tyson G, Nielsen P. Genome sequences of rare, uncultured bacteria obtained by differential coverage binning of multiple metagenomes. Nat Biotechnol. 2013;31. https://doi.org/10.1038/nbt.2480 PMID: 23302930

25. Chen S, Zhou Y, Chen Y, Gu J. fastp: an ultra-fast all-in-one FASTQ preprocessor. Bioinformatics. 2018; 34: i884–i890. https://doi.org/10.1093/bioinformatics/bty560 PMID: 30423086

26. Wick R. rrwick/Filtlong. 2020. Available: https://github.com/rrwick/Filtlong

27. Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, et al. SPAdes: A New Genome Assembly Algorithm and Its Applications to Single-Cell Sequencing. J Comput Biol. 2012; 19: 455–477. https://doi.org/10.1089/cmb.2012.0021 PMID: 22506599

28. Antipov D, Korobeynikov A, McLean JS, Pevzner PA. hybridSPAdes: an algorithm for hybrid assembly of short and long reads. Bioinforma Oxf Engl. 2016; 32: 1009–1015. https://doi.org/10.1093/bioinformatics/btv688 PMID: 26589280

29. Nurk S, Meleshko D, Korobeynikov A, Pevzner PA. metaSPAdes: a new versatile metagenomic assembler. Genome Res. 2017; 27: 824–834. https://doi.org/10.1101/gr.213959.116 PMID: 28298430

30. Kolmogorov M, Yuan J, Lin Y, Pevzner PA. Assembly of long, error-prone reads using repeat graphs. Nat Biotechnol. 2019; 37: 540–546. https://doi.org/10.1038/s41587-019-0072-8 PMID: 30936562

31. Kolmogorov M, Bickhart DM, Behsaz B, Gurevich A, Rayko M, Shin SB, et al. metaFlye: scalable long-read metagenome assembly using repeat graphs. Nat Methods. 2020; 17: 1103–1110. https://doi.org/10.1038/s41592-020-00971-x PMID: 33020656

32. Vaser R, Sović I, Nagarajan N, Šikić M. Fast and accurate de novo genome assembly from long uncorrected reads. Genome Res. 2017; 27: 737–746. https://doi.org/10.1101/gr.214270.116 PMID: 28100585

33. nanoporetech/medaka. Oxford Nanopore Technologies; 2020. Available: https://github.com/nanoporetech/medaka

34. Walker BJ, Abeel T, Shea T, Priest M, Abouelliel A, Sakthikumar S, et al. Pilon: an integrated tool for comprehensive microbial variant detection and genome assembly improvement. PloS One. 2014; 9: e112963. https://doi.org/10.1371/journal.pone.0112963 PMID: 25409509

35. Brandt C, Bongcam-Rudloff E, Müller B. Abundance Tracking by Long-Read Nanopore Sequencing of Complex Microbial Communities in Samples from 20 Different Biogas/Wastewater Plants. Appl Sci. 2020; 10: 7518. https://doi.org/10.3390/app10217518

36. Alneberg J, Bjarnason BS, Bruijn I de, Schirmer M, Quick J, Ijaz UZ, et al. Binning metagenomic contigs by coverage and composition. Nat Methods. 2014; 11: 1144–1146. https://doi.org/10.1038/nmeth.3103 PMID: 25218180

37. Wu Y-W, Tang Y-H, Tringe SG, Simmons BA, Singer SW. MaxBin: an automated binning method to recover individual genomes from metagenomes using an expectation-maximization algorithm. Microbiome. 2014; 2: 26. https://doi.org/10.1186/2049-2618-2-26 PMID: 25136443

38. Kang DD, Froula J, Egan R, Wang Z. MetaBAT, an efficient tool for accurately reconstructing single genomes from complex microbial communities. PeerJ. 2015; 3: e1165. https://doi.org/10.7717/peerj.1165 PMID: 26336640

39. Wick RR, Judd LM, Gorrie CL, Holt KE. Unicycler: Resolving bacterial genome assemblies from short and long sequencing reads. PLoS Comput Biol. 2017;13. https://doi.org/10.1371/journal.pcbi.1005595 PMID: 28594827

40. Wood DE, Salzberg SL. Kraken: ultrafast metagenomic sequence classification using exact alignments. Genome Biol. 2014; 15: R46. https://doi.org/10.1186/gb-2014-15-3-r46 PMID: 24580807

41. Menzel P, Ng KL, Krogh A. Fast and sensitive taxonomic classification for metagenomics with Kaiju. Nat Commun. 2016; 7: 11257. https://doi.org/10.1038/ncomms11257 PMID: 27071849

42. Kim D, Song L, Breitwieser FP, Salzberg SL. Centrifuge: rapid and sensitive classification of metagenomic sequences. Genome Res. 2016 [cited 3 Dec 2020]. https://doi.org/10.1101/gr.210641.116 PMID: 27852649

43. Marquet M, Hölzer M, Pletz MW, Viehweger A, Makarewicz O, Ehricht R, et al. What the Phage: A scalable workflow for the identification and analysis of phage sequences. bioRxiv. 2020. https://doi.org/10.1101/2020.07.24.219899

44. Zhu C, Miller M, Marpaka S, Vaysberg P, Rühlemann MC, Wu G, et al. Functional sequencing read annotation for high precision microbiome analysis. Nucleic Acids Res. 2018; 46: e23. https://doi.org/10.1093/nar/gkx1209 PMID: 29194524

45. Parks DH, Imelfort M, Skennerton CT, Hugenholtz P, Tyson GW. CheckM: assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes. Genome Res. 2015; 25: 1043–1055. https://doi.org/10.1101/gr.186072.114 PMID: 25977477

46. Brown C, Irber L. sourmash: a library for MinHash sketching of DNA. In: Journal of Open Source Software [Internet]. 14 Sep 2016 [cited 18 Nov 2019]. https://doi.org/10.21105/joss.00027

47. Parks DH, Chuvochina M, Waite DW, Rinke C, Skarshewski A, Chaumeil P-A, et al. A standardized bacterial taxonomy based on genome phylogeny substantially revises the tree of life. Nat Biotechnol. 2018; 36: 996–1004. https://doi.org/10.1038/nbt.4229 PMID: 30148503

48. Méric G, Wick RR, Watts SC, Holt KE, Inouye M. Correcting index databases improves metagenomic studies. bioRxiv. 2019; 712166. https://doi.org/10.1101/712166

49. Huerta-Cepas J, Forslund K, Coelho LP, Szklarczyk D, Jensen LJ, von Mering C, et al. Fast Genome-Wide Functional Annotation through Orthology Assignment by eggNOG-Mapper. Mol Biol Evol. 2017; 34: 2115–2122. https://doi.org/10.1093/molbev/msx148 PMID: 28460117

50. Huerta-Cepas J, Szklarczyk D, Heller D, Hernández-Plaza A, Forslund SK, Cook H, et al. eggNOG 5.0: a hierarchical, functionally and phylogenetically annotated orthology resource based on 5090 organisms and 2502 viruses. Nucleic Acids Res. 2019; 47: D309–D314. https://doi.org/10.1093/nar/gky1085 PMID: 30418610

51. Haas BJ, Papanicolaou A, Yassour M, Grabherr M, Blood PD, Bowden J, et al. De novo transcript sequence reconstruction from RNA-seq using the Trinity platform for reference generation and analysis. Nat Protoc. 2013; 8: 1494–1512. https://doi.org/10.1038/nprot.2013.084 PMID: 23845962

52. Patro R, Duggal G, Love MI, Irizarry RA, Kingsford C. Salmon provides fast and bias-aware quantification of transcript expression. Nat Methods. 2017; 14: 417–419. https://doi.org/10.1038/nmeth.4197 PMID: 28263959

53. Campanaro S, Treu L, Rodriguez-R LM, Kovalovszki A, Ziels RM, Maus I, et al. The anaerobic digestion microbiome: a collection of 1600 metagenome-assembled genomes shows high species diversity related to methane production. bioRxiv. 2019; 680553. https://doi.org/10.1101/680553

54. Wick RR, Holt KE. Benchmarking of long-read assemblers for prokaryote whole genome sequencing. F1000Research. 2020; 8: 2138. https://doi.org/10.12688/f1000research.21782.3 PMID: 31984131

55. Nicholls SM, Quick JC, Tang S, Loman NJ. Ultra-deep, long-read nanopore sequencing of mock microbial community standards. GigaScience. 2019;8. https://doi.org/10.1093/gigascience/giz043 PMID: 31089679

56. Lau MCY, Harris RL, Oh Y, Yi MJ, Behmard A, Onstott TC. Taxonomic and Functional Compositions Impacted by the Quality of Metatranscriptomic Assemblies. Front Microbiol. 2018;9. https://doi.org/10.3389/fmicb.2018.00009 PMID: 29387050