Comparing metagenomics and total RNA sequencing for taxonomic profiling of microbial communities in the context of freshwater assessments

Intro structure

1. Freshwater biomonitoring why and how; inclusion of microbes was recently advocated
2. Taxonomic identification via morphology and barcoding limited
3. Shotgun sequencing-based approaches such as metagenomics and metatranscriptomics overcome limitations, but which one is better suited (also pros and cons of both methods)?
4. What we did to compare both methods

# Introduction

PART 1 FRESHWATER ASSESSMENT WHY AND HOW

Freshwater ecosystems are valuable for economic productivity, ecosystem resilience, and for maintaining ecosystem services, including the supply of clean consumable water (Dudgeon et al., 2006). However, these ecosystems are heavily impacted by agricultural and industrial pollution, habitat fragmentation (wetland drainage, river straightening, and dam building), and the introduction of invasive species (Dextrase and Mandrak, 2006; Jensen et al., 2006). Consequently, freshwater ecosystems belong to the most threatened ecosystems with some of the highest rates of species loss (Malmqvist and Rundle, 2002).

To prevent this species loss, the natural status of freshwater ecosystems needs to be protected, preserved, and restored. Therefore, the ecological status of freshwater bodies must be evaluated, by generating an inventory of their biodiversity. Traditionally, animals, plants, and diatoms are in focus when generating such inventories. However, there are ongoing efforts to implement microbes as well, since they respond faster to environmental changes and therefore might better represent environmental conditions (Cordier et al., 2019; Foissner and Berger, 1996; McArthur, 2001; Pawlowski et al., 2016; Payne, 2013; Smith et al., 2015; Stoeck et al., 2018).

PART 2 LIMITATIONS

Biodiversity inventories are generated by taxonomically identifying the community of organisms in a sample. This process is called taxonomic profiling and traditionally done by assessing the morphology of organisms. However, morphological identification can be biased (Stein et al., 2014; Sweeney et al., 2011) or not feasible due to a lack of diagnostical traits, especially for microbes (Pawlowski et al., 2012; Will and Rubinoff, 2004). Consequently, DNA metabarcoding (Taberlet et al., 2012) has established as a complementing, DNA-based approach for taxonomic profiling of communities. DNA metabarcoding, however, can also implement bias, due to varying primer binding affinities (Alberdi et al., 2018; Elbrecht and Leese, 2015; Krehenwinkel et al., 2017; Piñol et al., 2014; Piper et al., 2019) and PCR-related bias (Jane et al., 2015; Nichols et al., 2018; Piper et al., 2019; Sze and Schloss, 2019; Taberlet et al., 1996). Furthermore, DNA metabarcoding primers are designed to target specific taxonomic groups, and taxonomically diverse communities cannot be entirely covered. Attempts to increase taxonomic coverage by including multiple primers failed because they introduced additional bias (Corse et al., 2019; De Barba et al., 2014; Piñol et al., 2019). Consequently, both traditional approaches and DNA metabarcoding are unsuitable for taxonomic profiling of the biodiversity in a freshwater ecosystem.

PART 3: METAGENOMICS AND METATRANSCRIPTOMICS

An alternative approach for taxonomic profiling of communities is shotgun sequencing. This approach involves the random fragmentation and sequencing of the entire DNA in a sample, which is called metagenomics, or the entire RNA in a sample, which is called metatranscriptomics. That way, entire communities can be sequenced and identified primer- and PCR-free and, therefore, excluding the bias associated with traditional approaches and DNA metabarcoding. Recent studies address advantages of implementing metagenomics and metatranscriptomics into freshwater assessments (Cordier et al., 2020; Leese et al., 2018). Although metatranscriptomics is usually applied for analyzing gene expression, in this study, we address potential advantages of metatranscriptomics over metagenomics for taxonomic profiling.

First, total RNA sequencing (total RNA-Seq), which represents metatranscriptomics without an mRNA enrichment step, naturally enriches sequencing data for common barcode sequences. This natural enrichment is achieved because >85% of RNA consists of rRNA (Maaløe, 1979) containing the common barcodes 16S, 18S rRNA, and 28S rRNA, whereas these barcodes can make up as little as 0.05% – 1.4% in metagenomics sequencing data (Logares et al., 2014; Yilmaz et al., 2011). In theory, this enrichment enables a better coverage of common barcodes with total RNA-Seq as compared to metagenomics, which should allow for more accurate taxonomic profiling of taxonomically diverse communities.

Second, metatranscriptomics can be used to identify the active part of a community (Geisen et al., 2015; Gomez-Silvan et al., 2018). In comparison, metagenomics targets the present community, including DNA of dead and/or inactive cells and extracellular DNA, which can make up over 90% of microbial DNA datasets (Carini et al., 2016; Torti et al., 2015). Information about the active community is desirable for ecological assessments as it reflects the portion of the community that is interacting with the environment and therefore better reflects environmental conditions.

PART 4: WHAT WE DO

To compare the performances of metagenomics and total RNA-Seq in taxonomic profiling accuracy, we applied metagenomics and total RNA-Seq to two sets of samples: 1) a commercially available microbial mock community and 2) fish tank water samples, which simulates environmental freshwater sampling. In addition, we applied a broad variety of common bioinformatic processing and analysis tools to both metagenomics and total RNA-Seq data of both sample sets to test impact of these tools on the taxonomic profiling accuracy.

Using the known composition of the mock community, we evaluated the accuracy of the two sequencing approaches and bioinformatic tools in terms of taxa and abundance recovery. This evaluation allowed us to determine the most accurate taxonomic profiling workflow, from sequencing to bioinformatical processing and analysis. We also showed how the taxonomic profile of the fish tank sample differed based on the applied sequencing approaches and bioinformatic tools. Finally, we assessed which tools in the bioinformatic workflow resulted in significant differences in the taxonomic profile for both sample sets.

# Methods:

The workflow of this study is shown in Fig. 1.

*Sampling*

The microbial mock community used in this study is commercially available as ZymoBIOMICS Microbial Community Standard II (Log Distribution) (Zymo Research; Cat # D6310; Irvine; CA U.S.A.). It consists of eight bacteria species (thee gram-negative and five gram-positive) and two yeast species. They are mixed to create log-distributed species abundances based on genomic DNA amounts (Tab. 1). The mock community is stored in DNA/RNA Shield (Zymo Research; Cat # R1100-50; Irvine; CA U.S.A.), which inactivates cells while preserving DNA and RNA. We generated three simulated water sample replicates by adding 130 µl of the microbial mock community to 50 mL ultrapure H2O three separate times.

Table 1: Microbial composition of the mock community (taken from the ZymoBIOMICS Microbial Community Standard II (Log Distribution) manual).

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Species | Defined Composition (%) | | | | |
| Genomic DNA | 16S Only1 | 16S & 18S1 | Genome Copy2 | Cell Number3 |
| *Listeria monocytogenes* | 89.1 | 95.9 | 91.9 | 94.8 | 94.9 |
| *Pseudomonas aeruginosa* | 8.9 | 2.8 | 2.7 | 4.2 | 4.2 |
| *Bacillus subtilis* | 0.89 | 1.2 | 1.1 | 0.7 | 0.7 |
| *Saccharomyces cerevisiae* | 0.89 | NA | 4.1 | 0.23 | 0.12 |
| *Escherichia coli* | 0.089 | 0.069 | 0.066 | 0.058 | 0.058 |
| *Salmonella enterica* | 0.089 | 0.07 | 0.067 | 0.059 | 0.059 |
| *Lactobacillus fermentum* | 0.0089 | 0.012 | 0.012 | 0.015 | 0.015 |
| *Enterococcus faecalis* | 0.00089 | 0.00067 | 0.00064 | 0.001 | 0.001 |
| *Cryptococcus neoformans* | 0.00089 | NA | 0.0014 | 0.00015 | 0.00007 |
| *Staphylococcus aureus* | 0.000089 | 0.0001 | 0.0001 | 0.0001 | 0.0001 |

1 The theoretical composition in terms of 16S (or 16S & 18S) rRNA gene abundance was calculated from theoretical genomic DNA composition with the following formula: 16S/18S copy number = total genomic DNA (g) × unit conversion constant (bp/g) / genome size (bp) × 16S/18S copy number per genome; 2 The theoretical composition in terms of genome copy number was calculated from theoretical genomic DNA composition with the following formula: genome copy number = total genomic DNA (g) × unit conversion constant (bp/g) / genome size (bp); 3 The theoretical composition in terms of cell number was calculated from theoretical genomic DNA composition with the following formula: cell number = total genomic DNA (g) × unit conversion constant (bp/g) / genome size (bp)/ploidy.

We also took 3 L of water from a fish tank containing soil, plants, roots, algae, fish, snails, and shrimp to simulate environmental freshwater sampling (Supplementary Fig. 1) using a bleach-sterilized and rinsed jug. The fish tank is in display at the Hagen Aqualab of the University of Guelph (Guelph; ON Canada).

*Laboratory processing*

We filtered all water samples in a clean laboratory (for details see Supplemental material 1). All samples were filtered through sterile 0.2 µm Nalgene Analytical Test Filter Funnels (Thermo Fisher Scientific; Cat # 145-2020; Burlington; ON Canada) using an 80 mbar Welch WOB-L® Dry Vacuum Pump (VWR International; Cat # 80077-612; Mississauga; ON Canada). We filtered the two types of samples on two different days. On the first day (31 Jan 2020), we filtered the three 50 mL microbial mock community/H2O mixes and added a negative filtration control by filtering 50 mL of ultrapure H2O along with the other samples. On the second day (05 Feb 2020), we filtered three times 1 L of the fish tank water an added a negative filtration control by treating an additional filter the same way as the other filters without actually filtering water. After filtration, we immediately cut each filter into small pieces and transferred them into ZR BashingBead Lysis Tubes (0.1 & 0.5 mm) (Zymo Research; Cat # S6012-50; Irvine; CA U.S.A.) which were prepared with 1 mL of DNA/RNA Shield under a clean hood in a low DNA-concentration laboratory prior to filtration.

We beat the BashingBead tubes on a Vortex-Genie 2 (Scientific Industries, Inc.; Cat # SI-0236; Burlington; NY U.S.A.) in a Horizontal-(24) Microtube holder (Scientific Industries, Inc.; Cat # SI‑H524; Burlington; NY U.S.A.) for 40 min at maximum rpm to break up cells. The manufacturer guaranteed reliable cell breakup of the purchased microbial mock community using this procedure.

For parallel DNA/RNA extraction from samples, we used a modified version of the Quick-DNA/RNA Microprep Plus Kit (Zymo Research; Cat # D7005; Irvine; CA U.S.A.), which was provided as a free sample by the manufacturer. We added a purification step using Zymo-Spin II‑µHRC Filters (Zymo Research; Cat # C1059-50; Irvine; CA U.S.A.) and modified the protocol to process more sample volume (for details see Supplemental material 2). We extracted the two types of samples on two different days under a clean hood in a low DNA-concentration laboratory and added a negative extraction control each day.

The extracted DNA and RNA along with the negative filtration and extraction controls were sent to Génome Québec (Montreal; QC Canada) for library preparation and shotgun sequencing. The DNA library was prepared with NEB Ultra II and the RNA library with NEB stranded total RNA library preparation while skipping the mRNA enrichment step to create a total RNA library. During library preparation, normalization was performed based on volume instead of concentration because we aimed for an equal relative sequencing depth between samples as compared to an equal total sequencing depth that might over- or underrepresent samples with high or low DNA/RNA amounts. The RNA library yielded fragments around 230 bp length, and to be able to compare DNA and RNA without choosing unnecessarily long paired-end reads, both libraries were sequenced on one Illumina MiSeq PE 150bp run.

*Bioinformatic processing*

﻿ All MinION nanopore filtered reads analyzed in this project were deposited in the NCBI SRA database (https://www.ncbi.nlm.nih.gov/sra). The data of the barcodes test run was deposited under Bioproject PRJNA627087 (samples accession numbers: SRR11581740 - SRR11581744). The data of the plastic and water samples was deposited under Bioproject PRJNA625720 (samples accession numbers: SRR11554946 - SRR11554965).

We obtained XXX paired-end sequences (SRA accession number: XXX), on average XXX per sample [how much will it vary because of our normalization method?], and processed them in five steps, using multiple common tools/parameters at each step (Fig. 2). For step one (trimming and quality filtering), we used Trimmomatic (Bolger et al., 2014) with four different quality cut-offs (﻿PHRED ≤2, ≤5, ≤10, and ≤20). For step two (rRNA sorting), we used four approaches: alignment-based – SortMeRNA (Kopylova et al., 2012), HMM-based – barrnap (Seemann, unpublished), kmer-based – rRNAFilter (Wang et al., 2017), and no sorting. All resulting reads after performing step one and two were separately pooled based on the two sample types and the pooled reads were used in step three (assembly), for which we used four DNA assemblers, SPAdes (Bankevich et al., 2012), metaSPAdes (Nurk et al., 2017), MEGAHIT (Li et al., 2015), IDBA-UD (Peng et al., 2012), and four RNA assemblers, Trinity (Grabherr et al., 2013), rnaSPAdes (Bushmanova et al., 2019), IDBA-tran (Peng et al., 2013) and Trans-ABySS (Robertson et al., 2010). For step four (mapping individual sample reads back to scaffolds), we used two programs, BWA (Li and Durbin, 2009) and Bowtie2 (Langmead and Salzberg, 2012). For step five (taxonomic classification), we used the reference databases NCBI nt (Agarwala et al., 2016), downloaded on 03 Feb 2020, and the reference database SILVA (Quast et al., 2013), downloaded on 16 Dec 2019. We used four approaches for the taxonomic classification: kraken2 (Wood et al., 2019), Centrifuge (Kim et al., 2016), BLAST (Altschul et al., 1990) with an E-value cut-off of e-05 and keeping the hit with the highest bitscore per sequence, and BLAST with an E-value cut-off of e-05 and filtering the hits with an in-house script (based on steps performed by the program CREST (Lanzén et al., 2012): filtering out hits below a bitscore of 155, only keeping hits within 2% of the best bitscore of each sequence, applying a cut-off for taxonomic ranks based on BLAST pident values, and identifying the LCA of each sequence).

To be able to compare the taxonomic classification results based on SILVA and NCBI nt, we standardized the taxonomy by translating the taxonomy of all SILVA hits into NCBI staxids using an in-house script.

We generated a pipeline for all combinations of steps and programs, and the full code with all program parameters and versions, as well as the scripts for translating SILVA taxonomy to NCBI staxids, are available on GitHub (XXX).

*Statistical evaluation*

XXX

* Give standardized information about the samples according to (Yilmaz et al. 2011) MIxS specifications

A picture containing toiletry, lotion

Description automatically generated

Figure 1: Study workflow



# Results

# Discussion:

Shotgun sequencing will highly favour microbes, and metazoans living in an ecosystem will be likely very underrepresented.

Point out that results are valid only for this specific sequencing depth. If sequencing depth increases, metagenomics will become more effective (because it doesn’t only cover rRNA barcodes but many more), but we don’t know to what degree/at what depth in relation to community complexity.

RNA hard to handle, short half-life, not feasible for biomonitoring?

Taxonomy-free biomonitoring

Total RNA Seq can be even further used for mRNA analysis – two birds with one stone

Role of mRNA-Seq for taxonomic profiling? (need to find REFS)

However, the application of metagenomics for taxonomic profiling is highly dependent on sequencing depth, i.e., the sequenced portion of a sample. This dependency is due to the small number of genes in an organism that are useful for taxonomic profiling. Standard barcode genes that are used for that purpose can make up as little as 0.05%-1.4% in metagenomics data (Logares et al., 2014; Yilmaz et al., 2011), and references for other genes are often missing, which is why the major portion of metagenomic data often remains unknown (Singer et al., 2020; Stat et al., 2017). The efficiency of metagenomics for taxonomic profiling is, therefore, dependant on the coverage of these barcode genes, which is directly related to the sequencing depth. The more complex a community is, the higher sequencing depth is required to cover barcode genes to an extend where the community can be accurately profiled. Consequently, given sufficient sequencing depth, metagenomics can outperform metabarcoding in terms of taxonomic profiling (Logares et al., 2014; Shah et al., 2010; Shakya et al., 2013), whereas at low sequencing depths, the coverage of barcodes can sometimes be too low and metagenomics can be outperformed by metabarcoding (Singer et al., 2020; Stat et al., 2017).

Another factor that needs to be considered for metagenomics is the high costs that are involved for high sequencing depths. Given the previously mentioned dependency of metagenomics on sequencing depth, it is recommended to aim for ﻿maximized sequencing output when performing metagenomics. ﻿Illumina HiSeq, NextSeq, and NovaSeq sequencing runs are considered to be suited for that purpose (Quince et al., 2017). However, running these sequencers is expensive, and for many biomonitoring applications, it is important to consider the limited budget available to stakeholders. Consequently, the use of these high sequencing output sequencers might not be affordable yet for routine biomonitoring.

We predicted that by using total RNA-Seq, we would be able to accurately profile the entire microbial mock community at a moderate sequencing depth and that this approach would outperform metagenomics. This would make total RNA-Seq attractive and affordable for stakeholders. We also predicted that significant differences would be observable for both the microbial mock community and the fish tank sample using different bioinformatic pipelines.

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# Supplemental material

A picture containing algae, broccoli, food, sitting

Description automatically generated

Supplementary Figure 1: Fish tank

**Supplemental material 1: filtration**

**Needed:**

* Zymo Bashing Bead tubes (ZR BashingBead Lysis Tubes (0.1 & 0.5 mm))
* DNA/RNA Shield reagent
* 0.2 µm Nalgene Analytical Test Filter Funnels
* Forceps, scissors, burner
* Bleach + EtOH

**Preparation:**

* Label bashing tubes (1 per sample + 1 filtration control) and load them with 1 mL of DNA/RNA Shield

**Procedure:**

1. Sterilize forceps and scissors with burner, then bleach, then EtOH before each filtration
2. Filter water sample, make sure to keep the filtration unit closed when opening the wrapping, to hold nothing but the sample over the unit, and to stand as far as possible away from the unit while it is open
3. Cut filter into small pieces (forceps and scissors) while filter lays on the filtration unit, make sure to stand as far as possible away from the unit
4. Transfer pieces into bashing tubes until all pieces are covered with DNA/RNA Shield
5. Clean filtration unit adapter with bleach after each filtration
6. Bashing tubes can be stored at -20°C

**Supplemental material 2: parallel DNA+RNA extraction from freshwater samples using the Quick-DNA/RNA Microprep Plus Kit and Zymo-Spin II-µHRC Filters (Zymo Research)**

*Note: maximum of 11 samples + extraction control possible at a time*

**Needed:**

* Access to a -80°C freezer
* Microcentrifuge
* Bead beater à  recommendation from Zymo Research is a Vortex Genie unit with a 24 Microtube holder
* ZymoBIOMICS Quick-DNA/RNA Microprep Plus Kit
* Zymo-Spin II-µHRC Filters
* 100% EtOH (1.5 mL per sample + extraction control)
* Eliminase (or other RNase-eliminating reagent)
* 11 microtube racks (1x Qubit tubes; 1x 2 mL tubes + samples + extra tubes; 2x 1.5 mL tubes; 3x columns; 4x collection tubes) + 1 rack for falcon tubes + 1 ice rack
* Timer

**Preparation (all one day in advance):**

*Note: 1 set refers to 1x the number of samples + extraction control*

* Clean whole extraction hood with bleach, EtOH, Eliminase (this order)
* Clean microtube and falcon racks in the same way and put in hood
* Clean pipettes, tip boxes (1x small, 1x medium, 3x large), and hood waste the same way
* Label tubes and columns in hood and close them after labelling (no detailed labels necessary unless specified)
  + 1 set 2 mL tubes
  + 1 set Zymo-Spin ICXM columns + collection tubes (labelled “DNA”)
  + 1 set Zymo-Spin IC columns + collection tubes (labelled “RNA”)
  + 9 more sets collection tubes (4 sets labelled “DNA”, 6 sets labelled “RNA”)
  + 2 sets 1.5 mL tubes (1 set labelled “DNA”, 1 set labelled “RNA”)
  + 2 sets Zymo-Spin III u-HCR Filter columns + collection tubes (1 set labelled “DNA”, 1 set labelled “RNA”)
  + 2 sets 1.5 mL tubes with detailed labels (1 set labelled “DNA”, 1 set labelled “RNA”)
  + 2 sets Qubit tubes (1 set labelled “DNA”, 1 set labelled “RNA”) + 4 additional Qubit tubes for standards (2 RNA + 2 DNA)
  + 2 additional 1.5 mL tubes for DNase-mix preparation + 2 additional 5 mL tubes for Qubit solution preparation (RNA + DNA)
* Put 100% EtOH and 5 mL tubes in falcon rack
* UV-sterilize everything overnight

**Procedure:**

1. Place filters/bead tubes (thawed) in Microtube holder on Vortex Genie, vortex for 40 min at max speed (never more than 18 tubes at a time, because >18 tubes will slow vortexing and create inaccurate results)
2. Centrifuge bead tubes for 1 min at 13,000 xg and room temperature
3. Transfer as much of the supernatant as possible into 2 mL tubes (ideally don’t transfer any beads)
4. Add 1 volume of **DNA/RNA Lysis Buffer** to samples and vortex

*Note: because we transferred all the supernatant from bead tubes (instead of 400 µl as specified in protocol), the samples are too large to carry out next steps in one go. The next steps have to be repeated, so keep all columns/tubes/mixes etc. First and second rounds are indicated in brackets.*

1. Transfer 800 µl (first round)/rest (second round) of samples into Zymo-Spin ICXM in a collection tube and centrifuge for 30 sec at 13,000 xg and room temperature. **SAFE THE FLOW-THROUGH**
2. Transfer Filter columns into a new collection tube
3. Add 1 volume of **100% EtOH** (800 µl (first round)/600 µl (second round)) to flow-through and mix well (pipette 10x up and down)
4. Transfer sample into Zymo-Spin IC columns in a collection tube and centrifuge for 30 sec at 13,000 xg and room temperature. (Note: Only 800 µl can be transferred at a time, so this step has to be repeated; repetition can be done right away by transferring the rest of the sample into the same columns in a new collection tube and repeating the centrifugation)
5. Transfer filter columns into a new collection tube

*Repeat steps 5-9 using the same columns.*

1. Remove all collection tubes and respective racks that are not needed anymore from hood
2. DNase treatment:
3. Prepare **DNase Reaction Mix**: for number of samples + extraction control + 1 buffer, mix 35 µl **DNA Digestion Buffer** with 5 µl **DNase I**
4. Wash all columns with 400 µl DNA/RNA Wash Buffer and centrifuge for 30 sec at 13,000 xg and room temperature
5. Transfer all columns in new collection tubes
6. Add 40 µl **DNase I Reaction Mix** directly to the column matrix
7. Incubate columns at room temperature for 15 minutes
8. Add 400 µl **DNA/RNA Prep Buffer** to columns and centrifuge for 30 sec at 13,000 xg and room temperature
9. Transfer all columns in new collection tubes
10. Add 700 µl **DNA/RNA Wash Buffer** to columns and centrifuge for 30 sec at 13,000 xg and room temperature
11. Transfer all columns in new collection tubes
12. Add 400 µl **DNA/RNA Wash Buffer** to columns and centrifuge for 2 min at 13,000 xg and room temperature to ensure compete removal of wash buffer
13. Transfer one column at a time into 1.5 mL tube (no detailed labels) and add 22.5 µl **ZymoBIOMICS DNase/RNase-Free Water** directly to column matrix, let stand for 5 minutes, and centrifuge for 30 sec at 13,000 xg and room temperature to elute DNA/RNA in water. (Note: only 8 open 1.5 mL tubes fit into 24-Microcentrifuge at a time)
14. Place Zymo-Spin II u-HRC Filter columns in new collection tubes and add 600 µl **ZymoBIOMICS HRC Prep Solution**. Centrifuge for 3 min at 8,000 xg and room temperature à “prepared filter columns”
15. Transfer one prepared filter column at a time into 1.5 mL tube (detailed labels), transfer eluted DNA/RNA into prepared filter column, and centrifuge for 3 min at 16,000 xg and room temperature. (Note: only 8 open 1.5 mL tubes fit into 24-Microcentrifuge at a time)
16. Aliquot 2.5 µl of each final sample into Qubit tubes for concentration measurement
17. Clean an ice rack with bleach, EtOH, Eliminase, and put final DNA/RNA samples on ice
18. Carry out Qubit measurement with 2 µl of aliquoted samples
19. Store final samples at -80°C