Journal of Microbiological Methods 114 (2015) 43–50

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Evaluation of water sampling methodologies for amplicon-based characterization of bacterial community structure

Christopher Staley [a], Trevor J. Gould [a,b], Ping Wang [a], Jane Phillips [b], James B. Cotner [c], Michael J. Sadowsky [a,d,*]

[a] BioTechnology Institute, University of Minnesota, St. Paul, MN, United States
[b] Biology Program, University of Minnesota, St. Paul, MN, United States
[c] Department of Ecology, Evolution and Behavior, University of Minnesota, St. Paul, MN, United States
[d] Department of Soil, Water and Climate, University of Minnesota, St. Paul, MN, United States

ARTICLE INFO

Article history:
Received 24 February 2015
Received in revised form 30 April 2015
Accepted 4 May 2015
Available online 6 May 2015

Keywords:
Bacterial community structure
DNA extraction kits
16S rDNA amplicons
Next-generation sequencing
Water volume

ABSTRACT

Reduction in costs of next-generation sequencing technologies has allowed unprecedented characterization of bacterial communities from environmental samples including aquatic ecosystems. However, the extent to which extrinsic factors including sampling volume, sample replication, DNA extraction kits, and sequencing target affect the community structure inferred are poorly explored. Here, triplicate 1, 2, and 6 L volume water samples from the Upper Mississippi River were processed to determine variation among replicates and sample volumes. Replicate variability significantly influenced differences in the community α-diversity ($P = 0.046$), while volume significantly changed β-diversity ($P = 0.037$). Differences in phylogenetic and taxonomic community structure differed both among triplicate samples and among the volumes filtered. Communities from 2 L and 6 L water samples showed similar clustering *via* discriminant analysis. To assess variation due to DNA extraction method, DNA was extracted from triplicate cell pellets from four sites along the Upper Mississippi River using the Epicentre Metagenomic DNA Isolation Kit for Water and MoBio PowerSoil kit. Operational taxonomic units representing ≤14% of sequence reads differed significantly among all sites and extraction kits used, although differences in diversity and community coverage were not significant ($P \geq 0.057$). Samples characterized using only the V6 region had significantly higher coverage and lower richness and α-diversity than those characterized using V4–V6 regions ($P < 0.001$). Triplicate sampling of at least 2 L of water provides robust representation of community variability, and these results indicate that DNA extraction kit and sequencing target displayed taxonomic biases that did not affect the overall biological conclusions drawn.

1. Introduction

Over the last two decades, characterization of microbial communities in aquatic ecosystems has received increasingly more attention, with next-generation sequencing (NGS) studies being rapidly exploited to characterize community shifts in response to global climate change and anthropogenic impacts (Drury et al., 2013; Fortunato et al., 2012; Newton et al., 2013; Staley et al., 2014, 2015; Zinger et al., 2012). Owing to the diversity and heterogeneity of these ecosystems, particularly rivers and streams, it has been presumed that there is a tradeoff between diversity estimation, requiring extensive sequencing and increased statistical power, and resolution of patterns in microbial biogeography between sites (Zinger et al., 2012). Standardized sampling methods have been argued against, due in large part to the possibility that differences in diversity between habitats may be artificially altered as a result of differences in heterogeneity (Cao et al., 2002). Increasing the community auto-similarity, the average similarity between replicate samples, by increasing the number of replicates or sample volume has been suggested to account for these differences in heterogeneity (Cao et al., 2002). Furthermore, the necessity of replication to evaluate statistical significance of results, especially when using "cutting-edge" methodologies, has been recently emphasized (Prosser, 2010).

The tradeoff between volume of water filtered and the number of replicates has historically been left to individual investigators (Zinger et al., 2012), where large sample volumes of tens of liters often limit the logistical capacity for replication. Prior characterization of a riverine bacterial community by DGGE fingerprinting found no differences in DGGE profile using volumes ranging from 35 to 1000 mL of water, with identical patterns between replicates for five samples processed in duplicate (Dorigo et al., 2006). However, a fluorescent *in situ* hybridization study of rare flavobacterial clades in seawater has also shown that increased sample volumes resulted in reduced variability in cell abundance values (Gómez-Pereira et al., 2010). Next-generation sequencing methods are revealing considerably greater diversity than previous techniques and have enabled the exploration of the 'rare biosphere' (Sogin et al., 2006), and relative abundances of taxa in

* Corresponding author at: BioTechnology Institute, University of Minnesota, 140 Gortner Lab, 1479 Gortner Ave, St. Paul, MN 55108, United States.
E-mail address: sadowsky@umn.edu (M.J. Sadowsky).

http://dx.doi.org/10.1016/j.mimet.2015.05.003
0167-7012/

these studies are similarly likely to be highly influenced by sample volume. To date, sample volumes in different amplicon-based NGS studies of freshwater and marine environments have been highly variable, ranging from 125 mL to 6 L of water (Fortunato et al., 2012; Ghai et al., 2011; Gilbert et al., 2009; Kolmakova et al., 2014; Portillo et al., 2012; Sogin et al., 2006; Staley et al., 2013). Comparisons of results among these studies are difficult due to differences in environment, filter pore size and type, and the hypervariable region of the 16S rDNA sequenced.

In order to deliver on the promise of NGS methods to nearly completely characterize microbial communities, these methodological parameters (*e.g.*, volume, filter pore size, and sequencing region) must be optimized for maximal coverage of the bacterial community. Studies of microbial communities in oligotrophic seawater environments have relied on using direct filtration of relatively large volumes (5 to 6 L) through 0.22-μm-pore-size filters (Gilbert et al., 2009), but more turbid riverine samples require pre-filtration to remove aggregates (Ghai et al., 2011; Staley et al., 2013). Furthermore, in highly turbid systems, such as the Mississippi River, filtration of large volumes through 0.22-μm filters is not logistically feasible, taking on the order of several hours to process a single sample, even after pre-filtration (Staley et al., 2013). Thus, community composition in these riverine environments may be biased by filtration volume and filter size. In addition, sequencing depth has been shown to highly impact the similarity between samples, where shallow sequencing results in greater variability among replicates (Gibbons et al., 2013; Staley et al., 2015). The need for normalization of NGS datasets to an equal number of samples for comparisons of α- and β-diversity has been recently discussed (Gihring et al., 2012), suggesting that studies will be limited by replicate sample(s) with the fewest numbers of sequences. To account for all of these sources of variability and potential limitations, sampling methods should be carefully established to minimize replicate variability and maximize community coverage.

DNA isolation methods have also long been shown to introduce bias when characterizing bacterial communities, due in large part to differences in resistance to cell lysis among species (von Wintzingerode et al., 1997). Pyrosequencing of mock communities extracted using different methods revealed method-specific biases in the relative abundance of taxa identified (Morgan et al., 2010). However, the magnitude of differences in community composition resulting from DNA extraction methods have yet to be analyzed for diverse, environmental communities. In addition, biases resulting from primer selection have been repeatedly demonstrated using NGS methods (Claesson et al., 2010; Youssef et al., 2009). Since the best taxonomic predictions are likely to result from full length 16S rDNA sequence analysis, it is reasonable to presume that increasing amplicon length will also improve taxonomic accuracy. However, there is evidence that declining sequence quality toward the ends of longer sequence reads may make assembly of paired ends difficult and introduce sequencing errors (Kozich et al., 2013). We postulate that these errors may result in artificially inflated estimates of bacterial diversity and inaccuracy in taxonomic assignment.

In this study, we assessed variability among replicate samples, at differing sample volumes, to determine the minimal volume required for precise characterization of community composition in a riverine ecosystem. Furthermore, communities characterized using two DNA extraction kits from different manufacturers, microorganisms captured on both (combined) 0.45-μm- and 0.22-μm-pore size filters, and sequence data obtained from either the V6 hypervariable region alone or the V4–V6 regions were compared to determine differences in inferred community composition, as well as ecological conclusions drawn. Power analysis was done for each of these experiments to determine whether sufficient replication and sequencing depth had been achieved. Results of this study suggest a robust and logistically feasible method for collection and processing of riverine water samples to optimize community coverage. Furthermore, current and emerging concerns regarding NGS sequencing and processing are addressed.

2. Materials and methods

2.1. Locations and sample collection

Samples for all experiments were collected from up to 11 sites along the Mississippi River in Minnesota from 2011 to 2014 (Supplementary Figure S1 and Table S1). Samples were collected for three independent experiments, the: 1) replicate/volume experiment assessing variation among replicates due to sample volume, 2) DNA extraction experiment assessing variation in community composition and β-diversity using different DNA extraction kits, and 3) 16S rDNA sequencing experiment assessing variation in community composition and β-diversity due to the amplicon sequenced. For the replicate/volume experiment, triplicate 20 L water samples were collected from the Twin Cities site in sterile 20 L carboys. For the DNA extraction experiment, two sets of triplicate 2 L samples were collected in sterile 2 L bottles at the Twin Cities, Minnesota River, Confluence, and Hastings sampling sites. For the 16S rDNA sequencing experiment, 40 L samples were collected in 20 L sterile carboys at all sampling sites, in both 2011 and 2012, as described previously (Staley et al., 2014).

2.2. Sample processing

For the replicate/volume and 16S rDNA sequencing experiments, water samples were filtered using a parallel, positive-pressure vacuum pump set-up, to efficiently pass through large volumes of water (Figure S2). As described previously (Staley et al., 2013), water samples were passed through four layers of sterile cheesecloth and then sequentially pumped through 90-mm-diameter P5 filters (Whatman, Inc., Piscataway, NJ, USA) and 142-mm-diameter, 0.45-μm-pore-sized, polyethane sulfonate filters (Pall Co., Port Washington, NY, USA). Filters were replaced as needed due to clogging. For each replicate collected in the replicate/volume experiment, after shaking by hand, sequential volumes of 1, 2, and 6 L were filtered (separate P5 and 0.45-μm-pore-sized filters were used for each volume), and the equipment was sterilized with 70% ethanol between volumes/replicates. For 16S rDNA sequencing, six to eight 0.45 μm filters were required. Each 0.45 μm, for both experiments, was cut in half using ethanol-sterilized scissors and each half was placed in a separate 50 mL conical tube for elutriation (see below).

A 2 L sterile filter flask connected to a vacuum line was used for the DNA extraction experiment. Water (2 L) was passed through a 45-mm-diameter, 5-μm-pore-sized nitrocellulose filter (MilliPore Corp., Bellerica, MA, USA). The filtrate was removed from the flask to the original collection bottle and then passed through a 0.45-μm-pore-sized mixed ester cellulose filter (MilliPore Corp.). The filtrate was removed to the original collection bottle, and finally filtered through a 0.22-μm-pore-sized nitrocellulose filter (MilliPore Corp.). For each size fraction, filters were replaced as needed due to clogging, and all filters for each pore size were placed in separate 50 mL conical tubes for elutriation (all filters representing a single pore size in a single tube). Cells were elutriated from filters by vortexing in 2 mL pyrophosphate (PP) buffer (0.1% sodium pyrophosphate, 0.2% Tween 20, pH 7.0) for 3 min. Cell suspensions were added to two 1.7 mL microcentrifuge tubes and pelleted by centrifugation at 13,000 $\times g$ for 3 min. The supernatant was discarded. Filters were vortexed a second time in 2 mL fresh PP buffer and cell suspensions were pelleted in the same microcentrifuge tubes already containing cell pellets with the supernatant again discarded. For replicate/volume and DNA extraction experiments, cell pellets from both tubes were combined, such that each cell pellet represented a single replicate, volume, and/or filter pore size. The 0.45-μm and 0.22-μm pellets were also combined for the DNA extraction experiment. For the 16S rDNA sequence experiment, multiple filters were elutriated in the same manner using six microcentrifuge tubes for all filters, such that cell pellets represented 6–7 L of water. Cell pellets were stored at −80 °C until DNA was extracted.