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Does universal 16S rRNA gene amplicon sequencing of environmental communities provide an accurate description of nitrifying guilds?

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Abstract

Universal (i.e., targeting most bacteria/prokaryotes) 16S rRNA gene based amplicon sequencing is widely used for assessing microbial communities due to its low cost, time efficiency, and ability to provide a full overview of the community. However, it is currently unclear if it can yield reliable information on specific microbial guilds, which can be obtained by using primer sets targeting functional genes or specific 16S rRNA gene sequences. Here, we compared the relative abundance, diversity, richness, and composition of selected guilds (nitrifiers), obtained from universal 16S rRNA gene based amplicon sequencing and from guild targeted approaches. The universal amplicon sequencing provided 1) accurate estimates of nitrifier composition, 2) clustering of the samples based on these compositions consistent with sample origin, 3) estimates of the relative abundance of the guilds correlated with those obtained from the targeted approaches and within ~1.2 orders of magnitude of them, but with measurable bias that should be considered when comparing estimates from both approaches. In contrast, the diversity and richness estimations using the universal 16S rRNA based amplicon sequencing were likely limited by the sequencing depth; therefore, we suggest preferring targeted approaches for assessing nitrifiers diversity and richness or using sequencing depth larger than those currently typically practiced.

Keywords: Diversity; Drinking water treatment; Wastewater treatment; Nitrifiers; AOB; Nitrospira.
1. Introduction

Community-wide high throughput amplicon sequencing of one or multiple hypervariable regions of the 16S rRNA gene has become a routine tool to describe the composition and diversity of microbial communities (Bartram et al., 2011; Caporaso et al., 2011). Beyond such an overall community assessment, it is often desirable to quantify and characterize specific constituent guilds in terms of abundance, composition, and diversity. For many guilds, the 16S rRNA gene can be informative because phylogenetic conservation of functional traits is common (Philippot et al., 2010; Martiny et al., 2013). In fact, if the microbial guild of interest consists of one or a few monophyletic clades (for example, ammonia-, nitrite-, or methane-oxidizers), it is possible to utilize 16S rRNA gene primers to specifically target the guild (Degrange and Bardin 1995; Kowalchuk et al., 1997; Hermansson & Lindgren, 2001; Graham et al., 2007). Additionally, if conserved signature functional genes exist, targeting them can be a strategy. For example, amoA, which codes for a subunit of the ammonia monooxygenase can be used to target ammonia oxidizing prokaryotes; and nxrB, which codes for the beta subunit of the nitrite oxidoreductase can be used to target nitrite oxidizing bacteria (NOB) (Norton, 2011; Pester et al., 2013). Targeting functional genes can be advantageous to access within-guild diversity, due to their high rate of evolution compared to the 16S rRNA gene (Dopheide et al. 2015).

In contrast to a non-specific approach such as universal (i.e., designed to target most bacteria) 16S rRNA gene amplicon sequencing, any guild-specific approach provides information only on the targeted guild but none on the rest of the community, which is an obvious limitation (Xue et al. 2013; Dopheide et al. 2015). The tradeoff is that universal approaches give a good overview of the whole
microbial community, but provide only limited information on non-dominant guilds because of their low contribution to the total sequence pool.

Therefore, it is currently unclear whether universal 16S rRNA gene amplicon sequencing is sufficient to obtain reliable information on specific microbial guilds and, more precisely, whether it correctly differentiates between samples with high and low guild diversity and whether it provides sufficient compositional information to identify samples with similar guild composition.

An additional concern regarding microbial community analysis using amplicon sequencing lies in its ability to provide reliable estimates of the relative abundance of the community constituents. Indeed, the quantitative nature of amplicon sequencing has been questioned (Zhou et al. 2011). Studies using mock communities have shown that the results from 16S rRNA gene based amplicon can be biased based on choice of primers and at different in-vitro (DNA extraction) and in-silico (sequencing and taxonomic assignment) experimental stages (Brooks et al. 2015; Parada et al. 2016; Thijs et al. 2017).

Despite known biases, 16S rRNA gene based amplicon sequencing has been used successfully for quantitative assessment of the ubiquitous taxa in the bacterial community (Ibarbalz et al. 2014). However, to our knowledge, the literature provides no assessment of the reliability of universal amplicon sequencing for quantifying specific guilds. Therefore, one of the aims of this study was to fill this gap.

Here, using nitrifiers as model guilds, we compared bacterial 16S rRNA gene amplicon sequencing (further referred to as ‘universal approach’) to a guild-targeted approach in their abilities to infer relative abundance, diversity, richness, and composition. Nitrifying microbial guilds are a key to ammonium oxidation in many natural and engineered ecosystems. They were traditionally strictly divided into ammonia-oxidizing prokaryotes and nitrite oxidizing bacteria (NOB) before the recent
discovery of *Nitrospira* types that can fully oxidize ammonia (Comamxon; Daims et al. 2015; van Kessel et al., 2015; Palomo et al. 2016). To target key nitrifying guilds, we used the functional genes *amoA* for ammonia oxidizing bacteria (AOB) and archaea (AOA); and *nxrB* for the main NOB genera *Nitrospira* and *Nitrobacter* (Arp and Stein 2003; Francis, Beman, and Kuypers 2007; Vanparys et al., 2007; Pester et al., 2013)

2. **Material and Methods**

2.1 **Biomass sampling and DNA extraction**

The biomass originated from the top (0-10 cm) layer of the after filter (AF) of 4 drinking water treatment plants (DWTP) in Denmark and from the nitrifying reactor (NR) of wastewater treatment plants (WWTP) from Denmark and Sweden and from an anammox reactor (Sjolunda (AR)) in a WWTP from Sweden (Figure S1). Three samples for each plant were used for qPCR. For the 16S rRNA gene amplicon sequencing, five samples were analyzed for three DWTP (Glostrup, Hillerod, and Odense (AF)) and single samples for all other plants.

DNA was extracted from 0.5 g of wet drained sand from DWTP and 0.5 g sludge from WWTP using FastDNA™ spin kit for soil (MP Biomedicals, Solon, OH, USA) according to manufacturer’s instructions. Duplicate DNA extractions were done for each sample. DNA concentration was estimated using NanoDrop ND-1000 UV-VIS Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and the extracts were stored at -20 °C until further analysis.

2.2 **qPCR, PCR and amplicon sequencing**

Quantitative PCR (qPCR) analyses were conducted on a Chromo4 thermocycler using Opticon Monitor version 3 (BioRad). Each qPCR reaction contained 12.5 µL of 2X iQ SYBR green Supermix (Bio-Rad
Laboratories, Hercules, California, United States), 500 mM primer, DNA template (10 ng), and DNA/RNA-free water (Qiagen, Hilden, Germany) to 25µL. For each sample, DNA with average concentration ~26 and ~68 ng/µl for sand and sludge samples respectively was sent for 16S rRNA gene, amoA, and nxrB PCR, purification and amplicon sequencing (2 x 300 nucleotides) using the Illumina MiSeq platform at the DTU Multi Assay Core Center (Kgs. Lyngby, DK). For the universal 16S rRNA gene sequencing, primers (Bakt_341F and Bakt_805R, spanning the V3-V4 regions) and PCR conditions were from Herlemann et al., 2011. For amoA AOB sequencing and qPCR, primers (amoA-1F and amoA-2R primers) and PCR conditions were from Rotthauwe et. al., 1997. For nxrB Nitrospira sequencing and qPCR, primers (nxrB169f and nxrB638r primers) and PCR conditions were from Pester et al., 2013. Primers (CTO189fA-B, CTO189fC, and RT1r) and PCR conditions for AOB 16S rRNA gene qPCR were from Kowalchuk et al., 1997. QPCR efficiencies and correlation coefficients obtained from the standard curves are in Table S1.

2.3 Bioinformatics analysis

Sequences generated as paired FASTQ files were processed using DADA2 (Version - 1.4; Callahan et al., 2016). DADA2 was preferred over other clustering-based methods as it recovers sequence variants that can vary by as little as a single nucleotide and thus avoids aggregation of sequences at arbitrary identity cutoff. DADA2 was used for quality filtering, trimming, de-replicating the reads, for inferring sequence variation through default error model parameters, for merging paired reads, for removing chimera, and for assigning taxonomy using Silva reference database v123 for 16S rRNA gene and custom taxonomy files for amoA and nxrB. A very similar pipeline was used for amoA and nxrB, except that for amoA only the forward reads (237 bp after trimming for quality) were analyzed due to the amplicon length. We applied frameshift correction (from package DECIPHER version 2.6; Wright
2016) and discarded sequences that were too distant from our reference sequences. The data obtained from DADA2 was analyzed using Phyloseq R Package (Version - 1.7.12; McMurdie and Holmes 2013). Raw sequence files were deposited into the sequence read archive at GenBank under the study accession number SRP127282.

2.4 Relative abundance estimation of AOB and *Nitrospira*

Universal 16S rRNA gene amplicon sequencing data was used to perform CaRcone analysis to obtain the average 16S rRNA gene copies per genome in the amplicon libraries (R script [https://github.com/ardagulay/CaRcone---Community-average-rRNA-gene-copy-nr-estimator](https://github.com/ardagulay/CaRcone---Community-average-rRNA-gene-copy-nr-estimator)). The total numbers of bacteria per gram of biomass were derived by normalizing the gene copy number from qPCR with 1.75 16S rRNA gene copies per genome obtained from the CaRcone analysis. The abundance of AOB and *Nitrospira* cells per gram of biomass was obtained by assuming 2 *amoA* and 2 *nxrB* copies per genome (McTavish, Fuchs, and Hooper 1993; Lücker et al. 2010). Thus, comparing *nxrB* for *Nitrospira* and *amoA* for AOB with 16S rRNA gene was feasible as the abundance values obtained from two genes were normalized per genome.

The relative fractions of AOB and *Nitrospira* were calculated by dividing the normalized abundance estimates obtained from *amoA* and *nxrB* based qPCR by that obtained from 16S rRNA gene based total bacteria qPCR. Similarly, AOB and *Nitrospira* abundance estimates obtained from *amoA* and *nxrB* based sequencing were compared to their respective values obtained after dividing reads assigned to AOB and *Nitrospira* in the 16S rRNA gene based total bacteria sequencing by the total number of reads.
2.5 Statistical analysis

The alpha diversity metrics, Shannon diversity and observed richness were calculated using the ‘estimate_richness’ function in the Phyloseq R package version (Version - 1.7.12; McMurdie and Holmes, 2013). The estimates of Shannon diversity after rarefaction were calculated by performing rarefaction using the minimal number of sequences in each gene library, as we would lose a large amount of sequences from \textit{amoA} and \textit{nxrB} libraries if we rarified to the minimum number of sequences assigned to AOB and \textit{Nitrospira} from the 16S rRNA gene library. For phylogenetic diversity calculation, first the sequences were aligned using the ‘AlignSeqs’ function in the DECIPHER R package (Version 2.0.2; Wright, 2015), then a neighbor joining (NJ) tree was constructed using phangorn R package (Version 2.3.1; Schliep, 2011), phylogenetic diversity (Faith’s PD; the sum of the branches of the phylogenetic tree) was then calculated using the PhyloMeasures R package (Version 2.1; Faith, 1992; Tsirogiannis and Sandel, 2016).

To statistically compare the relative abundance and diversity estimates from universal and targeted approaches we used major axis (MA) regression (a Type II regression model) because for both type of estimates none of the variables (x and y) were experimentally controlled (Pierre Legendre and Legendre 1988). As the relative abundance estimates were homoscedastic on the log scale, we performed the regression analysis on the log scale with average abundance values from each plant. The MA regression was performed using ‘lmodel2’ package version 1.7-3 in R (P Legendre 2018).

To evaluate whether the universal and targeted approaches separated the samples similarly based on nitrifier composition, Principal Coordinate Analysis (PCoA) of samples based on their nitrifier composition obtained from universal and targeted approaches was performed separately. The PCoA
ordination matrix based on Bray Curtis similarities was generated using ‘ordinate’ function and the
PCoA plot was generated using ‘plot_ordination’ function in the Phyloseq R package (Version -
1.7.12; McMurdie and Holmes, 2013). Further, Procrustes analysis was performed to compare the
multidimensional shapes of the two PCoAs by transforming them into a state of best
superimposition (Peres-Neto and Jackson 2001). The significance of the similarity of the two PCoA
matrices was then tested by a procrustean randomized test (PROTEST) that determines whether the
sum of the residual nonconformities was less than that expected by chance (Jackson, 1995).

The clustering analysis was performed using the K-medoids clustering, a partitioning method that
clusters a set of objects into K clusters, with K set based on a priori knowledge (Jin and Han 2010).
K medoids clustering method was applied using PAM clustering in vegan (version 2.4-3) and
cluster (version 2.0.6) R packages (Dixon 2003; Mächler et al., 2012). The Clustering analysis
figures were generated using ‘clusplot’ function in cluster R package (version 2.0.6; Mächler et al.,
2012) and further edited using Inkscape (version 0.921; Bah, 2007).

3. Results and discussion

We investigated biomass extracted from three nitrifying and one anammox wastewater treatment
bioreactors and from four biological rapid sand filters producing potable water (Figure S1). At both
types of plants, nitrifiers perform a fundamental role in ammonium removal and are known to range
from 1% to 10% in abundance relative to the whole microbial community (Wagner et al., 2002; Gülay
et al., 2016; Tatari et al., 2017).

In the universal approach, sequences assigned to nitrifier guilds were extracted to estimate the relative
abundance, diversity, richness, and composition of nitrifying guilds (Table S2). These estimates were
then compared to those obtained from the guild-targeted approaches (qPCR for relative abundance and amplicon sequencing for diversity and composition; Table S2).

_Nitrobacter_ (NOB) and ammonia-oxidizing archaea (AOA) were minor fractions of the nitrifiers in the analyzed communities. Indeed, both types of nitrifiers were undetected in the universal approach; the amplification of _Nitrobacter nxrB_ prior to amplicon sequencing failed; and AOA were below or close to the detection limit of the _amoA_ qPCR assay (10 gene copies per reaction) in WWTP and DWTP, respectively (data not shown). Therefore, here we focus only on AOB and _Nitrospira_; further collectively referred to as nitrifiers.

In the universal approach, 20,372 and 346,879 sequences were assigned to AOB and _Nitrospira_, respectively, across all sampling sites. From the targeted approach, 501,381 sequences were assigned to AOB (_amoA_) and 2,165,787 to _Nitrospira_ (_nxrB_) (Table S3).

### 3.1 Relative abundance of nitrifiers

Targeted quantification of AOB was performed by qPCR using two primer sets targeting either betaproteobacterial-AOB-specific 16S rRNA gene or _amoA_. The relative abundances obtained from the universal approach were always lower than the 16S rRNA gene based qPCR (Figure 1A) but higher than the _amoA_ qPCR (Figure S2-A). These results are consistent with previous observations from environments similar to those investigated in this study indicating that the 16S rRNA gene AOB primers from Kowalchuk et al., 1997, being unspecific, tend to overestimate AOB abundance, and that the _amoA_ primers can underestimate AOB abundance depending upon the AOB composition (Figure S2-B; Dechesne et al. 2016; Tatari et al., 2017). Nitrifier composition for this study is presented and discussed in further sections. Compared to the universal approach, the _nxrB_ qPCR yielded slightly higher relative abundance estimates for _Nitrospira_ in all WWTP but lower for all DWTP (Figure 1B).
These observations for DWTP are consistent with previous findings of *Nirespira* relative abundance comparison of 16S rRNA gene based qPCR specific to *Nitrospira* and universal amplicon sequencing (Gülay et al. 2016). Overall, for both of the guilds, even if we observed inconsistency between the targeted and the universal approaches, the universal approach based estimates were always within ~1.2 orders of magnitude of the targeted approaches.

Using MA regression analysis, we explored next whether the estimates from both approaches were linearly related. For AOB, the slope was significantly different from 0 (p value 0.003) and very close to 1 (slope estimate = 0.9), suggesting a direct proportionality between the approaches. However, the intercept was negative (p value <0.05), consistent with the observation made earlier that the 16S rRNA gene based AOB quantification by qPCR tend to be significantly higher than the one based on universal amplicon sequencing (Figure 1-A). For *Nitrospira*, the intercept was not statistically different from 0 and the slope was significantly higher than 1 (slope estimate = 2.2) suggesting that the *Nitrospira* quantification based on universal amplicon sequencing increases more rapidly than *nxrB* qPCR with *Nitrospira* relative abundance (Figure 1-B).

Overall, the estimates of relative abundance of both guilds (AOB and *Nitrospira*) from universal approach correlated with those obtained from the targeted approaches and were within ~1.2 orders of magnitude of them, but with measurable bias. The universal approach certainly provides useful quantitative information for AOB and *Nitrospira* but the highlighted biases should be considered when comparing estimates from universal and targeted approaches for these guilds.
Figure 1 (color in print): Comparison of the relative abundance of AOB (A) and *Nitrospira* (B) based on universal (y-axis) and targeted (x-axis) approach. Universal 16S rRNA gene based amplicon sequencing was compared with qPCR (target: 16S rRNA gene for AOB in panel A and *nxrB* for *Nitrospira* in panel B) for four after filters (AF) from DWTP, three nitrifying reactors (NR) and one anammox reactor from WWTP. The error bars represent the standard deviation for each plant.

### 3.2 Alpha diversity of nitrifiers

For both guilds and irrespective of the metric used, the targeted approach always resulted in higher alpha diversity values compared to the universal approach (Figure 2). We ascribe this observation to the facts that the functional gene libraries contained approximately ∼24 fold (AOB) and ∼6 fold (*Nitrospira*) more sequences than retrieved from the universal sequencing (Table S3) and that diversity estimates can be highly reliant on the depth of sequencing (number of sequences per sample; Caporaso et al., 2011; Gihring et al., 2012; Smith and Peay, 2014). Additionally, the rate of evolution of functional genes (*amoA, nxrB, rpoB* etc.) is known to be higher than that of ribosomal genes (16S rRNA).
For both guilds, the Shannon diversity and observed richness calculated from the universal approach correlated very poorly with that estimated from the targeted approach (Figure 2 A, B, D). This was also true when these indexes were calculated after rarefaction to the minimal number of sequences in each comparison (Figures S3 and S4). Phylogenetic diversity (Faith’s PD; the sum of the branches of the phylogenetic tree) for AOB and observed richness (without rarefaction) for *Nitrospira* were the only diversity indices with a significantly positive correlation between both approaches (p value 0.009; Figure 2C and p value 0.01; Figure 2E). This frequent inconsistency between targeted and universal approach suggests that the guild-targeted approaches should be preferred to estimate the diversity of AOB and *Nitrospira*. 

rRNA gene), which makes them more phylogenetically resolutive (Case et al., 2007; Pester et al., 2012; Pester et al., 2013). Thus, using the targeted approach on functional genes results in the detection of more sequence variants than with the universal approach, which translates to higher but also, likely more accurate, diversity estimates.
Figure 2 (color in print): Comparison of alpha diversity (Shannon; A and D), observed richness (B and E) and phylogenetic diversity (Faith’s PD; C and F) of AOB and *Nitrospira* based on universal (16S rRNA gene) and targeted (*amoA*-top and *nxrB*-bottom) approaches. Scales on x and y-axis are different. The black line is the major axis regression and the respective p values are mentioned in each panel suffixed with a star sign if significant. For *Nitrospira*, a sample for which zero values were obtained with the universal approach (Sjolunda (AR)) was excluded from the linear regression. Likewise, for one DWTP, no valid AOB *amoA* sequences were obtained (Table S3).
3.3 Composition of nitrifiers

3.3.1 AOB composition

The universal approach only yielded sequences assigned to cluster 6 (represented by *Nitrosomonas aestuarii* and *Nitrosomonas marina*) and 7 (represented by *Nitrosomonas europaea* and *Nitrosomonas eutropha* strains) whereas the targeted approach also detected clusters 0, 2, 3, (*Nitrosospira*) and 8 (represented by *Nitrosomonas nitrosa*; Figure S5). Both approaches identified the same dominant cluster (Cluster 6) in five out of seven sites. Only for one plant (Sjolunda) did both approaches provide the exact same picture of the cluster-level composition. For all DWTP and one WWTP, the universal approach only showed the presence of cluster 6; whereas the targeted approach indicated the presence of additional clusters: cluster 7 in all these plants, plus cluster 0, 2, 3 and 8 in one WWTP (Kallby; Figure S5). These observations relate with the low relative abundance of AOB observed earlier by universal 16S rRNA gene sequencing (Figure 1A) as clusters other than cluster 6 were largely unmapped for four out of seven sites by the universal approach due to their very low relative fraction to the total community.

Finally, at the subcluster taxonomic resolution, we found that all samples where cluster 6 was predominant consisted mainly of subcluster 6A members (represented by *Nitrosomonas oligotropha* and *Nitrosomonas* sp. ls79A3; Figure S6). As mentioned in previous studies, *amoA* AOB based primers (from Rotthauwe et al., 1997), having been designed primarily from cluster 7 members sequences, underestimate AOB abundance when subcluster 6A members are dominant (Dechesne et al., 2016). This likely explains why *amoA* based qPCR underestimated AOB in these plants (Figure S2).
3.3.2 *Nitrospira* composition

*Nitrospira* composition inferred from both approaches was largely similar for all sites (Figure S7). Both approaches identified strong compositional differences between DWTP and WWTP, the former being dominated by lineage 2 and the later by lineage 1 (Figure S7).

Overall, the universal approach successfully identified the dominant clusters/lineages within AOB and *Nitrospira* for most plants. Therefore, the universal approach can be a preferred choice for estimating the composition of these guilds, especially when the focus is on dominant guild members.

3.4 Samples ordination and clustering based on nitrifiers composition

In the PCoA analysis, both approaches separated DWTP and WWTP samples for both the guilds (Figure 3) but the PCoA plots obtained from the targeted approaches were visually different from that generated from their universal approach counterpart (Figure 3). Procrustean randomized test (PROTEST) was not significant for both guilds (AOB: p value = 0.8; *Nitrospira*: p value = 0.5), confirming the visual differences as significant.

Next, K-medoids clustering was performed to evaluate how the two approaches grouped (clustered) the samples based on their nitrifier composition. Here, we set K as the number of plants because we assumed that samples originating from the same plant should cluster together because communities from similar ecological sites tend to be similar in composition (Whittaker 1965).
Figure 3 (color in print): Clustering analysis of samples after PCoA ordination of their compositions of AOB (panels A & B) and Nitrospira (panels C & D) obtained from the targeted and universal approach. Shaded ellipses followed by cluster number represent the clusters. Each solid circle and triangle is a sample, with color coding according to the plant. Seven replicate samples were used for Odense and Glostrup, three for Hillerod and one for all other plants. For AOB, the two components explain 50.4% of the variability for the PCoA based on amoA (A) and 57.3% for 16S rRNA gene (B). For Nitrospira the two components explain 67.5% of the variability for the PCoA based on nxrB (C) and 71.3% for 16S rRNA gene (D). The number of clusters (K) were adjusted to 7 and 8 for AOB and Nitrospira, respectively, according to the number of plants sampled.
3.4.1 Sample clustering based on AOB composition

The universal approach separated the samples into seven clusters corresponding to the seven plants, except for one replicate which was misplaced (Figure 3B). amoA incorrectly distributed the DWTP samples into four clusters, with one cluster comprising samples from all three plants (Figure 3A). Samples from two WWTP were placed in the same cluster (Cluster 2; figure 3A) whereas they were separated by the 16S rRNA gene (Cluster 2 and 4; figure 3B). Therefore, it is apparent that the targeted approach did not provide better clustering than the universal approach.

3.4.2 Sample clustering based on *Nitrospira* composition

The universal and targeted approaches for *Nitrospira* clustered samples similarly with some minor variations (Figure 3 C and D). For example, nxrB based analysis separated all plants except for one cluster which contained samples from two DWTP and one WWTP (Cluster 1; figure 3C). For the universal approach, all plants were separated except for one sample from a DWTP that clustered with samples from another DWTP (Cluster 6; figure 3D). Two WWTP that were separated (Cluster 6 and 8; figure 3C) by the targeted approach were now clustered together (Cluster 3; figure 3D). Here, the universal and the targeted approaches thus gave similar clustering outputs.

4. Conclusion

Taken together, our results provide the first systematic validation of the use of universal approaches for quantification and composition estimation of nitrifying guilds, as done by several authors (Rughöft et al., 2016; Ramanathan et al. 2017; Meerbergen et al., 2017; Saarenheimo et al. 2017). Overall, the 16S rRNA gene based universal amplicon sequencing of environmental communities can be efficiently used as substitute to more narrow targeted approaches for:

1) The assessment of nitrifier composition, as it captured the main ecological signal (the most dominant taxa) for all plants.
2) Sample clustering based on nitrifier composition, as it correctly separated samples from different plants.

The universal approach also provided relative abundance estimates within ~1.2 orders of magnitude of those from the targeted approaches, but with systematic biases that should be considered especially when comparing quantification estimates from both approaches.

Lastly, for the universal approach, diversity estimation was likely limited by the sequencing depth. Therefore, we suggest preferring targeted approaches for assessing nitrifiers diversity, unless much higher sequencing depths than commonly currently practiced are used.

Conflicts of interest
The authors declare no conflicts of interest.

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