Targeted metagenomics using probe capture detect a larger diversity of nitrogen and methane cycling genes in complex microbial communities than traditional metagenomics

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Manuscript contribution to the field

Metagenomic sequencing often yields limited numbers of sequences of rare microbial taxa or functional genes, preventing in-depth analyses of specific populations and functional groups. Amplicon-based approaches enable the higher diversity coverage of target populations, but the

drawback is the difficulty in designing unbiased primers that cover the highest intra-group diversity. Targeted metagenomics overcomes these challenges and results in similar community structure as traditional amplicon sequencing, while expanding the sequence space in a less biased metagenomic-based approach. Therefore, targeted metagenomics is an invaluable tool for studying the diversity of specific populations within complex natural microbiomes. Here, we present and evaluate a probe library designed for targeted metagenomics of nitrogen and methane cycling genes in complex communities.

Abstract

Microorganisms are key players in the global cycling of nitrogen (N) and carbon (C), controlling their availability and fluxes, including the emissions of the powerful greenhouse gases nitrous oxide (N_2O) and methane (CH_4). Characterizing the microbial functional guilds driving these processes is crucial for understanding ecosystem functioning and predicting their responses to environmental changes. Standard sequence-based characterization methods often reveal only a limited fraction of their diversity in nature because of their low relative abundance, the insufficient sequencing depth of traditional metagenomes of complex communities, and limitations in coverage of PCR-based assays. Here, we developed and tested a targeted metagenomics approach based on probe capture and hybridization to simultaneously characterize the diversity of multiple key metabolic genes involved in inorganic N and CH_4 cycling. We designed comprehensive probe libraries for each of the 14 target marker genes comprising 264,000 unique probes. These probes were used to selectively enrich the target genes in shotgun metagenomic libraries. In validation experiments with the mock communities of known microorganisms, targeted metagenomics yielded gene profiles similar to those of the original communities. Only GC content had a small effect on probe efficiency, as low GC targets were less efficiently detected than those with high GC, within the mock communities. Furthermore, the relative abundances of the marker genes obtained using targeted or traditional shotgun metagenomics from agricultural and wetland soils were significantly correlated, indicating that the targeted approach did not introduce significant quantitative bias. In addition, using archaeal *amoA* genes as a case-study, targeted metagenomics identified substantially higher taxonomic diversity and a larger number of sequence reads per sample, yielding diversity estimates 28 or 1.24 times higher than shotgun metagenomics or

amplicon sequencing, respectively. Notably, shotgun metagenomics detected only three out of the 84 *amoA* gene phylotypes detected using targeted metagenomics. Our results show that targeted metagenomics complements current approaches to characterize key microbial populations and functional guilds in biogeochemical cycles in different ecosystems, enabling more detailed, simultaneous characterization of multiple functional genes.

Introduction

The global nitrogen (N) and carbon (C) cycles are essential processes of the Earth's biosphere and crucial for ecosystem functioning (Zaehle 2013, Gruber and Galloway, 2008; Archer 2010; Falkowski et al., 2000; Steffen et al. 2015). All major N transformation processes (i.e., nitrogen fixation, nitrification, denitrification, dissimilatory nitrate reduction to ammonium (DNRA), and anaerobic ammonium oxidation, or anammox), are performed exclusively by the functional guilds of bacteria, archaea, and eukaryotes (Galloway et al., 2004; Stein and Klotz, 2016; Kuypers et al., 2018; Offre et al. 2013). Their activities regulate N availability for primary producers and microorganisms across ecosystems and control the production and consumption of the potent greenhouse gas nitrous oxide (N_2O) and other gaseous N compounds through processes such as nitrification, denitrification, and non-denitrifier N_2O reduction (Barnard, et al., 2005, Thompson et al., 2012; Jones et al., 2014; Prosser et al. 2020). Methane (CH_4) is another major product of the microbial trophic chain underlying C cycling, and, like the most inorganic N compounds, its production and consumption are also regulated by highly specific groups of microorganisms (Bodelier and Laanbroek 2004, Murrel and Jetten 2009, Conrad 2009). Importantly, CH_4 is the second most potent greenhouse gas after carbon dioxide (CO_2) and, together with N_2O, contributes at least 25% of the total global warming caused by greenhouse gases (Myhre et al., 2013; Friedlingstein et al., 2020; Lan et al., 2023).

Despite their important ecological role, microorganisms participating in inorganic N and CH_4 transformations typically constitute a small fraction of microbial communities in most soil, sediment, and aquatic ecosystems (Håvelsrud, et al., 2011; Palmer et al., 2012; Aanderud et al., 2015, Nelson et al, 2016, Ouygang and Norton 2020). Community profiling based on 16S rRNA

genes only allows for very limited information about microbial functions (Alteio et al. 2021). Even in the rare cases when functions can be inferred from organism phylogeny, the striking genetic and functional diversity within functional guilds remains concealed, such as that among their key metabolic enzymes (e.g., ammonia monooxygenase (*amoA*), Alves et al. 2018; methyl co-enzyme M reductase (*mcrA*), Borrel, 2019). Additionally, taxonomy-based approaches are inadequate in elucidating processes that have a broader taxonomic distribution than originally thought (e.g. Kuypers et al. 2018, Borrel 2019, Li et al. 2023; Saghaï et al., 2024). Shotgun metagenomics is the typical method of choice for obtaining a relatively unbiased picture of the natural microbiome, provided that issues with sample preparation, DNA extraction, library preparation, and sequencing method can be ruled out (Pan et al., 2010, Nnadozie, Lin and Govinden, 2015; Sinha et al., 2017; Sui et al., 2020). Nevertheless, in complex and diverse microbial communities such as those in soils and sediments, even deeply-sequenced metagenomes do not uncover the full diversity of microbial functional guilds and key functional genes owing to their typically low relative abundances within the broader community (Palmer et al., 2012, Orellana et al. 2019, Ouygang and Norton 2020, Zhao et al. 2023). As a low-cost alternative, the characterization of specific functional groups has long relied on the PCR amplification of genes that encode key metabolic enzymes, as for example, of organisms involved in various N and CH_4 cycling pathways (Knief 2015; Brauer et al. 2021; Alves et al. 2018; Romdhane et al. 2021, Clark et al. 2022). However, the gene diversity captured by gene-specific PCR assays is limited by the primers used, which introduce biases that make comparisons among different genes or samples difficult (Hallin and Lindgren, 1999; Throbäck al. 2004; Kolb et al., 2003; Siljanen et al., 2011). Furthermore, in complex microbial communities, target genes of interest are encoded by a large diversity of organisms, and usually include a large fraction of novel gene sequence variants from uncharacterized organisms (Jones et al. 2014; Saghaï et al 2023). Thus the geometric expansion of genomic data has made it increasingly obvious that designing a single PCR primer pair to target the inherent diversity of metabolic genes is highly problematic (Bonilla-Rosso et al. 2016). Therefore, linking ecosystem functions to microbial communities is often only possible through the complex and resource-intensive combinations of meta-omics and isotope labelling approaches (Tveit et al. 2015, Orellana et al. 2019).

126 Targeted high-throughput sequencing approaches, known as probe capture, also called
127 hybridization capture, hybridization-based target enrichment, or captured metagenomics, have
128 been used to study complex eukaryotic samples such as human exons, ancient human genomes,
129 plant transcriptomes, and cancer marker single nucleotide polymorphisms (Skoglund et al. 2014,
130 Ichida et al. 2019, Futema et al. 2012, Gasc et al., 2016, Kamil et al. 2021, Bewicke-Copley et al.
131 2019). A few studies have also used this approach to facilitate the in-depth study of microbial
132 communities using either 16S rRNA genes or other microbial functional genes (Denonfoux et al.,
133 2013; Manoharan et al. 2015, Noyes et al., 2017, Beaudry et al., 2021). The probe capture approach
134 relies on targeting specific short sequences within a broader genomic pool (e.g., full genomes,
135 genome or gene fragments) with biotin-labelled probes. The probes that hybridize with their target
136 regions are then selectively captured from the full genomic library using streptavidin-labelled
137 magnetic beads (Liu et al., 2016). Different from sequence capture, where multiple probes are
138 designed to cover a whole exon, captured metagenomics relies on the large databases of multiple
139 target genes clustered at predefined similarity cut-offs, which are used to design a large number of
140 generic probes that are able to capture an extended sequence space for each of these sequence
141 clusters (Kushwaha et al., 2015, Manoharan et al., 2015).
142
143 To improve detection and the characterization of important, but low abundant microbial guilds
144 involved in N and CH_4 cycling in natural communities, we developed and evaluated a probe-based
145 targeted metagenomics approach to key genes involved in these processes. In addition to the
146 enhanced resolution and coverage of genetic sequence space, this approach allows the parallel
147 analysis of distinct steps within N- and CH_4- cycling pathways, thus combining the advantages of
148 both shotgun metagenomics and amplicon-based approaches. Here, we present a new probe set for
149 targeting 14 distinct functional genes involved in inorganic N cycling (i.e., N fixation, nitrification,
150 denitrification, anammox, and DNRA), and three genes involved in CH_4 production and
151 consumption. We evaluated our approach using mock communities comprising of DNA from
152 microorganisms involved in inorganic N cycling, or CH_4 production or consumption with varying
153 gene GC% content, which is known to affect probe hybridization efficiency. As a proof-of-concept
154 for complex communities, we performed shotgun metagenomics and targeted metagenomics on
155 agricultural and wetland soil samples and compared the diversity of two functional markers, the

156 archaeal *amoA* genes (marker for ammonia oxidizers) and *nosZ* genes (marker for nitrous oxide
157 reduction).
158
159 **Materials and methods**
160
161 **Construction of the target gene databases (TDBs)**
162 Target gene databases (TDBs) were constructed, containing all identifiable variants for the
163 following key genes: the nitrogenase iron subunit (*nifH*), bacterial and archaeal ammonia
164 monooxygenase subunit A (*amoA)*, nitrite oxidoreductase beta subunit (*nxrB)*, hydrazine
165 oxidoreductase A (*hzoA)*, formate dependent nitrite reductase (*nrfA*), periplasmic nitrate reductase
166 alpha subunit (*napA*), respiratory nitrate reductase alpha subunit (*narG*), copper-containing nitrite
167 reductase (*nirK*), cytochrome cd_1 nitrite reductase (*nirS*), nitric oxide reductase subunit B (*norB*),
168 nitrous oxide reductase (*nosZ*), particulate methane monooxygenase subunit A (*pmoA*), soluble
169 methane monooxygenase component A alpha chain (*mmoX*), and methyl-coenzyme M reductase
170 I subunit alpha (*mcrA*).
171
172 Hidden Markov Model (HMM) models were generated in order to identify all variants of the target
173 genes from public databases (Fig. 1). These models were built based on reference sequence
174 alignments from curated databases already available for selected genes, such as *amoA* (Alves et
175 al., 2018), *pmoA* (Knief et al., 2015), *nosZ* (Jones et al., 2013), *nirK, nirS, nor* (Graf et al., 2014);
176 and the Fungene repository (Fish et al., 2013). For target genes where alignments were not
177 available, reference alignments were generated from gene sequence data publicly available on the
178 NCBI WGS-database, from the full length open-reading frame of each subunit, to cover all known
179 diversity in each gene. Structure-based searches of the Genbank nt- and envnt- database were
180 subsequently performed with nhmmer using the generated HMMs for every target gene (Wheeler
181 & Eddy, 2013) on a local supercomputer cluster (Centre of scientific computing CSC, Espoo,
182 Finland) (July 2017). The HMM models are available through Zenodo:
183 https://doi.org/10.5281/zenodo.15276905 (In script: probe-capture/2-
184 selected_outputs/hmmer_profiles.zip The specificity of HMM searches was evaluated by
185 comparing the results to the latest curated sequence databases of selected genes (Alves et al., 2018;
186 Graf et al., 2022, with primary data obtained directly from the respective authors), and results were

187 further evaluated based on annotation and gene structure. This sequence selection process
188 generated ~600,000 unique sequences across all gene families. The obtained database was
189 clustered to 100% identity in order to remove duplicates sequences in database with cd-hit and
190 manually inspected to exclude 16S rRNA genes. The final output comprised the target gene
191 database (TDB; available through Zenodo: https://doi.org/10.5281/zenodo.15276905 (In script:
192 probe-capture/2-
193 selected_outputs/all_genes_compiled_ncbi_nt_envnt_fungene_split_file_0.fasta.gz (four files
194 ..file_0-3.fasta.gz)). The workflow can be seen in Fig 1. Scripts for Target gene database search
195 is available in Zenodo (/probe-capture/tree/main/3-
196 other_scripts/Henri_scripts/Script_probe_capture.sh).
197
198 **Generation of probes for target genes**
199 The MetCap bioinformatics pipeline was used as a protocol to produce probes from highly
200 complex datasets for targeted metagenomics (Kushwaha et al., 2015). Default parameters were
201 used for designing up to six unique 50mer probes for each sequence cluster in the TDBs (clustered
202 with an 80% identity clustering threshold), with melting temperature of 47 °C, resulting in a set of
203 263,111 unique gene-specific probes (Fig 1, and in Zenodo: probe-capture/2-
204 selected_outputs/Final_N_probes.list1.fasta). The probes were synthesized by NimbleGen SeqCap
205 EZ (Roche NimbleGen, Inc., Madison, USA) as Custom design (since the MetCap pipeline was
206 used for probe design, probe order was done with Roche HyperDesign tool:
207 www.hyperdesign.com), with biotin labelling to enable retrieval of the hybridized targets using
208 streptavidin coated magnetic beads (Kushwaha et al., 2015; Manoharan et al., 2015).
209
210 **Extraction of DNA from cultures and generation of mock community DNA samples**
211 DNA samples from mock communities encoding the genes of interest were generated by mixing
212 genomic DNA from different organisms in variable proportions, in order to simulate variation in
213 overall GC% content. The pool of DNA in the mock communities included DNA from:
214 *Nitrosospira multiformis*, *Nitrososphaera viennensis*, *Nitrospira defluvii*, Ca. *Kuenenia*
215 *stuttgartiensis* PCR fragment of the full-lenght *hzoA* gene, *Pseudomonas aeruginosa*, *Escherichia*
216 *coli*, *Shigella sonnei*, *Cupriavidus metallidurans*, *Cupriavidus necator*, *Dyadobacter fermentans*,
217 *Pseudomonas stutzeri*, *Rhodobacter sphaeroides*, *Salinibacter ruber*, *Sulfurimonas denitrificans*,

218 *Methylosinus trichosporium* Ob3b, *Methylocella tundrae*, *Methylomicrobium buryatense*,
219 *Methanoregula boonei*, and *Methanolacinia petrolearia*. Strain information, including genome or
220 fragment size and the number of target genes per organism are shown in Supplementary Table S1.
221 DNA from cultured organisms was extracted from 1% SDS-treated cell pellets in CTAB buffer,
222 followed by phenol:chloroform:isoamyl alcohol extraction and ethanol precipitation, as described
223 before (Jones et al., 2013). The relative abundances of the microorganisms in the mock
224 communities were multiplied with the median genome GC% contents to generate a weighted GC%
225 content of the pool of samples with the following values: 47, 50, 53, 57, 60, and 63 (Supplementary
226 Table S2). Pooling ratios of microorganisms' DNA were calculated based on the expected number
227 of functional genes in the extracted DNA, the DNA concentration (determined with a Qubit HS
228 dsDNA kit (Thermo)) and genome size. Relative gene abundances in mock communities were
229 calculated and compared to the sequences generated using targeted metagenomics. The relative
230 abundance of each gene was calculated against the sum of abundance values of all 14 genes in the
231 targeted metagenomes of the mock community samples (relative abundance of a specific gene =
232 100 x (gene reads / sum of reads of 14 genes)). The captured reads for mock community targeted
233 metagenomics are shown in Table S4.
234
235 **Annotation of coding sequences from the mock community dataset**
236 Predicted proteomes from the mock communities were retrieved from NCBI to count the number
237 of each functional genes per genome. For *Nitrospira defluvii*; NC_014355.1, genes were predicted
238 from the genome nucleotide sequence using Prodigal (v2.6.3) (Hyatt et al., 2010). Proteins were
239 annotated using KOFAM (HMM database of KEGG Orthologs). Each protein was assigned the
240 top hitting KO. Where available, cut-offs provided with KOFAMscan (Aramaki et al., 2020) were
241 used to filter spurious results.
242
243 **Extraction of DNA and determination of chemical parameters from environmental samples**
244 Samples from two different environments, an agricultural soil in Hungary (n = 3), and a wetland
245 in Bellefontaine, France (n = 3) (Table S3), were collected in order to assess the effectiveness of
246 the probe set in enriching functional genes in different sample contexts. Extraction of DNA from
247 the environmental samples was performed as previously described by Siljanen et al., (2019).
248 Briefly, samples were homogenized by bead-beating 0.5 g of soil at a speed of 5.5 m/s, for 30 sec

249 with phenol:chloroform:isoamyl alcohol extraction in CTAB buffer, followed by ethanol
250 precipitation. The quality of DNA extracts was assessed using a NanoDrop ND-1000 (Thermo),
251 and DNA concentration was measured with a Qubit HS dsDNA kit (Thermo). The soil chemical
252 parameters soil C/N ratio, pH, Fe II/III-, ammonium- and nitrate content were determined as
253 described earlier (Siljanen et al., 2019; Bagnoud et al, 2020).
254
255 **Targeted metagenomic library preparation, target enrichment of libraries and sequencing**
256 **with Illumina Miseq**
257 For each sample, sequencing indexes and sequencing adapters were provided as commercial
258 service by the Centre for Genomic Research (CGR) -laboratories, at the University of Liverpool,
259 Liverpool, UK. Libraries were produced with KAPA HyperPlus Library Preparation (Roche) kit
260 to produce insert sizes of 630 bp according to the manufacturer's instructions. The protocol is
261 shown in Fig. 1. and described here briefly: 150ng of environmental DNA was fragmented with
262 KAPA fragmentation buffer and KAPA Frag Enzyme at +37°C for 20min. Fragmented DNA was
263 ligated to SeqCap Adapters A and B, +20°C for 15 minutes. Fragments were purified 0.65x
264 volumes of AMPure XP Reagent for 5 min at room temperature in magnetic particle collector
265 (MPC). Then the clear supernatant was removed and the library was washed twice with 200 µl of
266 80% ethanol. The libraries were removed from MPC by eluting them to 53 µl of elution buffer (10
267 mM Tris-HCl, pH 8.0) by incubated two minutes. Each library was amplified for 7 cycles, with
268 KAPA HyperPlus Library Preparation kit in order to add the indexes for each sample (cycling
269 conditions: +98°C, 15 sec; 60° for 30 sec; +72°C for 30 sec). Amplified libraries were purified
270 with AMPure XP beads as above.
271 The hybridization reactions to the NimbleGen SeqCap EZ Developer Probe set were performed
272 according to the manufacturer's instructions. The hybridization was done in non-binding DNA
273 background 25 µg, on top of 1 µg of each library equally pooled Multiplexed DNA Sample Library
274 were added. The Multiplexed Hybridization Enhancing Oligo Pool (2,000 pmol in 2µl volume),
275 the SeqCap HE Universal Oligo (1,000 pmol in 1µl volume) and (1,000 pmol in 1µl volume)
276 SeqCap HE Index Oligo Pool) was mixed with 1 µg of equally pooled Multiplexed DNA Sample
277 Library. Two volumes of AMPure XP Reagent was added on top of the above mixture and
278 thoroughly mixed. Samples were incubated for 10 minutes to allow the sample library to bind to
279 the beads. Samples were placed on the MPC to capture the beads and the solution allowed to clear.

280 Once clear, the supernatant was discarded carefully, to not disturb the beads. Afterwards 190 µl of
281 80% ethanol was added to the samples containing the bead-bound DNA samples. The samples
282 were left on the MPC during this step. Samples were incubated at room temperature for >30
283 seconds. The 80% ethanol was carefully removed and discarded, without disturbing the beads.
284 Beads were dried at room temperature with the tube lid open for 5 minutes (or until dry).
285 A master mix of the following reagents was prepared, scaling up to reflect the number of captures:
286 7.5 µl of the 2x Hybridization Buffer, 3 µl of Hybridization Component A. Then 10.5 µL of the
287 Hybridization Buffer/Hybridization Component A mix from the previous step was added to the
288 bead-bound DNA sample. Samples were removed from the MPC and mixed thoroughly. It was
289 important that enough mixing was performed at this step to yield a homogeneous mixture. It was
290 left to sit at room temperature for 2 minutes. Sampled were then placed on the MPC. After liquid
291 cleared, 10.5 µL of supernatant (entire volume) was removed and placed in a new tube containing
292 4.5 µl of the SeqCap EZ Developer Probe pool. This was mixed thoroughly. Hybridization
293 incubation was performed in a thermocycler using the following program with heated lid set to 10
294 °C above the block temperature: incubation was 95°C for 5min, and 47°C for 72 hours (SeqCap
295 EZ HyperCap Workflow protocol, Manoharan et al., 2015).
296 To purify the hybridized libraries the SeqCap Pure Capture Bead Kit was used. To prepare the
297 beads for binding the samples, 50 µl of beads (streptavidin coated magnetic beads) was added to
298 the 1.5 ml tube at room temperature for capturing reaction. Tubes were placed on a MPC, the
299 supernatant removed and beads were washed two times with 100 µl of 1x Bead Wash Buffer.
300 Afterwards it was removed from the MPC and mixed by pipetting up and down. Beads were bound
301 on the magnetic particle collector. Once the liquid was clear, the supernatant was removed. In the
302 second wash, the beads were aliquoted with 50 µl of 1X Bead Washing Buffer. Beads were bound
303 and the supernatant again removed. One hybridization sample (15 µl volume) was added to the
304 washed SeqCap Pure Capture Beads. Samples were bound to the beads by placing the samples in
305 a thermocycler at +47°C for 15 minutes (heated lid at +57°C). Then 100 µl of 1x Wash Buffer was
306 added on top of the Capture Beads and bead-bound DNA and mixed thoroughly. The tube was
307 placed on the MPC to capture the beads. The solution was allowed to clear, and the supernatant
308 removed, while being careful not to disturb the beads. Afterwards, 200 µl of 1x Stringent Wash
309 Buffer was added to each capture reaction. Those were mixed by pipetting up and down, when

310 tubes were not in a MPC. Samples were placed in the thermocycler to +47°C for 5 minutes. The
311 wash was repeated with 1x Stringent Wash Buffer. Next, 200 µl of 1x Wash Buffer I was added,
312 vortexed for 10 second, and incubated 1 minute at room temperature. Once the solution was clear,
313 it was removed, again being careful not to disturb the beads. The washing with 1X Wash buffer II
314 was repeated, and then 1x with Washing Buffer III. Samples were removed from the MPC and 53
315 µl of PCR-grade water was added to the bead-bound sample. Sampled were then incubated at room
316 temperature for 2 minutes and placed in the MPC. Then 50 µl of clear solution was collected in a
317 new tube.
318
319 Sequencing for probe hybridized and washed DNA was performed with Illumina MiSeq PE300
320 chemistry in the Centre for Genomic Research (CGR), University of Liverpool, Liverpool, UK,
321 resulting in 198,700-311,500 reads per sample for the environmental samples and up to 2,600,000
322 reads for the mock communities.
323
324 **Targeted metagenomics read processing and mapping**
325 All six possible reading frames of the nucleotide sequence reads generated by the targeted
326 metagenomic sequencing of the mock community samples were translated using transeq from the
327 EMBOSS package (v6.6.0.0) (Rice et al., 2000). These were mapped to the genomes using
328 DIAMOND blastp (v2.0.6.144) (Buchfink et al., 2015), with a minimum percentage identity of
329 60%. This threshold was selected as it resulted in the largest percentage of mapped reads while
330 being stringent enough to prevent spurious mappings. No minimum coverage was used to account
331 for reads that did not fully overlap with the proteins from the mock community dataset. For each
332 direction, up to four matches were retained, to account for cases where a single read spanned
333 neighboring proteins. Multiple matches in the same direction could overlap by up to 15 amino
334 acids to account for possible protein fusions or sequencing errors. The blastx searches against the
335 *refseq* database (Altschul et al., 1990) were performed in order to remove duplicates or reannotate
336 falsely annotated entries due to the presence of homologous gene families in our dataset (*amoA*
337 vs. *pmoA*, *nxrB* vs. *narH* nitrate reductase or *napA*+*narG* vs. *nrfA*). The blastx search was done to
338 evaluate whether the read was correct or not, and the run was made with the default parameters of
339 blast. Each of the (up to four) matches were used to assign KOs to the reads, based on the predicted

340 KOs of the proteins to which they mapped. If, in a given direction, a read was assigned the same
341 KO multiple times, this was collapsed into a single hit, retaining the hit with the best E-value.
342
343 **Evaluation of functional annotation of reads**
344 Protein predictions for each read were performed using nhmmer from the HMMER suite
345 (v.3.3)(Eddy 2011) using the in-house generated HMMs for target genes as well as KOFAM as
346 described above. An inclusion threshold of <0.0001 was used when assigning protein predictions
347 for the reads. For a given read, using all six of the translated frames, up to four protein assignments
348 could be made, evaluated based on KO bit scores or identity filtering. Reads assigned KOs for the
349 function of interest were counted as positively identified hits. Subsequently, reads were mapped
350 to their corresponding coding sequences (CDS) from the mock community organisms to determine
351 which reads constituted "true positives" (TP) or "true negatives" (TN). Reads mapped to a given
352 CDS were assigned functions according to the above-mentioned KOFAM CDS annotation as their
353 "true function". If the HMM assignment (both in-house and from KOFAM) of a read matched the
354 functional assignment of the CDS to which it was mapped, then it was counted as a TP. The TP,
355 false positive (FP), and false negative (FN) values were summed up for all models using all
356 methods, and precision and recall were calculated. Read mapping (described above) was used to
357 determine the total number of possible TPs of the reads (Fig. S6), from which the actual number
358 of TPs was subtracted to calculate the number of FNs. Precision was calculated as the ratio of TPs
359 to total positive detections (i.e., number of TPs and FPs). Recall was calculated as the ratio of TPs
360 to all theoretically obtainable true positives (i.e., sum of TPs and FNs). The script is available on
361 Zenodo: https://doi.org/10.5281/zenodo.15276905 (/probe-capture/tree/main/3-
362 other_scripts/Angus_scripts).
363
364 **Shotgun metagenomics library preparation, sequencing with Illumina HiSeq and analysis**
365 Shotgun metagenomic sequencing was performed on environmental DNA (n = 3) from agricultural
366 soil and wetland soil (Table S3), extracted as described above for TDB production. Illumina
367 TruSeq library preparation with an insert size of 250bp was carried out with 1 µg of DNA and
368 sequencing was performed in Illumina HiSeq PE150 lane as a commercial service in Vienna
369 Biocenter Core Facility (VBCF, Austria) and Microsynth AG, Switzerland. In this approach, the
370 previously generated HMM profiles were used to identify the target gene pools (E < 0.001). The

371 predicted functions of each identified sequence were confirmed by tblastx against the *refseq*
372 database, using DIAMOND (Buchfink et al., 2015). Annotation and identity information was
373 further processed with awk in unix and in Rversion 3.5.3 to produce lists of community structures
374 for each target gene. The relative abundance of each gene was normalized against the sum of total
375 reads, Table S4: Relative abundance of gene x = (Abundance of gene x / total reads sequenced) x
376 100.
377
378
379 ***amoA* gene amplicon sequencing**
380 Archaeal *amoA* gene amplicons from environmental DNA (n = 3) from the agricultural soil were
381 generated as described earlier by Siljanen et al., (2019), and sequencing was performed with
382 Illumina MiSeq PE250, in LGC, Münich, Germany. In brief, *amoA* gene amplicons were generated
383 using 0.6 ng DNA template with GoTaq DNA polymerase (Promega) and 0.5 µM primers
384 (CamoA_19F [5'-ATGGTCTGGYTWAGACG-3'] and TamoA_632R-4 [5'-
385 GCKGCCATCCATCKRTANGTCCA-3']) with a 5'-prime Illumina sequencing adaptor [5'-
386 CTCTTTCCCTACACGACGCTCTTCCGATCT-3'] for both end of above primers for making
387 the indexing possible in the sequencing service laboratory. Cycling conditions were the following:
388 1min initial denaturation at 95°C, 30sec denaturation at 94°C, 30sec annealing at 60°C and 45sec
389 extension at 72°C, with 35 cycles. PCR reactions were performed in triplicate for each sample (n
390 = 3), and purified with the High Pure PCR product purification kit (Roche). Sequencing indexes
391 were added by LGC, where libraries were equally pooled and sequenced with Illumina MiSeq
392 PE250.
393
394 **Processing of amplicon *amoA* reads**
395 Archaeal *amoA* (Thaumarchaeal-*amoA*, *TamoA*) amplicon reads processing was carried out by a
396 custom-based Python script available at Zenodo: https://doi.org/10.5281/zenodo.15276905;
397 /probe-capture/tree/main/1-scripts/1-amplicon_seq_script_v1.sh -folder). Briefly, quality
398 trimming of sequencing reads was performed by truncating amplicon forward read R1 reads to
399 200bp and reverse read R2 180bp, discarding all reads with an expected error greater than 0.5. The
400 *amoA* reads were dereplicated and amplicon sequence variants (ASVs) were assigned to taxonomic
401 bins described in the reference *amoA* gene database (Alves et al. 2018) using a 55% identity cutoff

402 with USEARCH8 (Edgar, 2010). Chimeras were filtered out with UCHIME (Edgar, 2011). For
403 the remaining ASVs, quality controlled R1 and R2 reads were fused together, classified with
404 UCLUST in QIIME (Caparaso et al., 2010) and clustered to OTUs with 97% sequence identity
405 cut-off for each taxonomic bins. Taxonomic bins of each *TamoA* gene cluster were generated using
406 the reference gene database and taxonomy by Alves et al. (2018) (Altschul et al. 1990; Callahan
407 et al., 2016). Relative abundance of *TamoA* taxonomic bins was calculated as follows: Relative
408 abundance of *TamoA* taxonomic bin x = (Abundance of *TamoA* taxonomix bin x / total *TamoA*
409 reads) x 100. In Fig. 4A, the relative abundance of each taxonomic bin (relative to the column
410 sum) was plotted for each sample. The sequencing depth for the *TamoA* gene amplicon library was
411 ~27,000-58,000 PE250 reads per sample up to 31Mb for the agricultural soil (Table S4).
412
413 **Processing of metagenomic *amoA* reads**
414 Archaeal *amoA* (Thaumarchaeal-*amoA*, *TamoA*) reads from targeted- and shotgun metagenomics,
415 were identified with the *TamoA* in-house HMM model. The forward reads were dereplicated and
416 assigned to the reference *amoA* gene database using a 55% identity cutoff with USEARCH8
417 (Edgar 2010). Chimeras were filtered out with UCHIME (Edgar 2011), all reads were clustered
418 with UCLUST with 97% sequence identity in QIIME and assigned to taxonomic bins based on the
419 closest *amoA* genes from the taxonomy database of OTUs (Alves et al. 2018) (Caporaso 2010).
420 Relative abundance was calculated as follows: Relative abundance of *TamoA* taxonomic bin x =
421 (Abundance of *TamoA* taxonomix bin x / total *TamoA* reads) x 100. In the targeted metagenomics
422 approach, multiple detection of the same taxonomic bin is possible because of six probes are used
423 for each sequence cluster. However, when relative abundance for each detected taxonomic bin is
424 calculated for targeted metagenomic, if multiple detection occurs in the relative abundance
425 calculation the community composition is balanced because of equal number of probes per each
426 sequence cluster. Therefore, the relative abundance of different sequencing methods can be
427 compared. The sequencing depth for the *TamoA* gene for three shotgun metagenomics *TamoA*
428 reads out of ~22-58M PE150 reads per sample up to 17 Gb, and for targeted metagenomics 4472-
429 8230 *TamoA* reads out of ~220-311k PE300 reads per sample up to 160 Mb, for the agricultural
430 soil (Table S4). The metagenomic reads processing is described in the custom-made script
431 available at Zenodo: https://doi.org/10.5281/zenodo.15276905 (targeted with probe-capture:

432 tree/main/1-scripts/2-probe_capture_script_v1.sh, and shotgun metagenomics: tree/main/1-
433 scripts/3-metagenomics_script_v1.sh).
434
435 **Phylogenetic placement analysis of *nosZ* reads from shotgun and targeted metagenomics**
436 **libraries.**
437 A reference alignment and phylogeny for *nosZ* was generated from full length *nosZ* amino acid
438 sequences obtained from genomes downloaded from the NCBI genome database (accessed
439 October 2019). A profile HMM for *nosZ* was generated from a previously published *nosZ* dataset,
440 as described above (Graf et al, 2022). Reads were converted to amino acid sequences and then
441 aligned using *hmmalign* within the HMMer suite (v.3.1.2)(Eddy 2011), and the homologous
442 sections were identified using FastTree (v. 2.1.11, Price et al., 2010). The reference protein
443 alignment was used for phylogenetic reconstruction using IQ-TREE (v1.6.12) with best model
444 selection and 1000 ultrafast bootstraps. The Le-Gascuel (LG) substitution model with 10 rate
445 categories was selected after automatic model selection. For the phylogenetic placement of *nosZ*
446 reads obtained using either shotgun metagenome sequencing (n=3) or targeted metagenomics
447 (n=3) of the agricultural site, the relevant reads were pooled together and clustered with CD-HIT
448 (Fu et al., 2012) at 90% sequence identity before translating and aligning to the reference *nosZ*
449 alignment using *hmmalign*. The translated reads were then placed in the reference phylogeny using
450 the next generation evolutionary placement algorithm (EPA-NG v0.3.8; Barbera et al., 2019), with
451 the same model parameters as used for constructing the reference phylogeny. The placement
452 positions with the highest likelihood weight ratio were plotted on the reference phylogeny in R
453 using the 'ggtree' package (v3.4.2, Yu et al., 2016). Script available for nosZ phylogenetic
454 placements at Zenodo: https://doi.org/10.5281/zenodo.15276905 (/probe-capture/tree/main/3-
455 other_scripts/Chris_Jones_scripts).
456
457 **Data and script availability**
458 The shotgun metagenomic, targeted metagenomic and *amoA* amplicon sequencing data have been
459 deposited in NCBI SRA under the Bioproject numbers PRJNA898102 and PRJNA488558. All
460 scripts are available at Zenodo: https://doi.org/10.5281/zenodo.15276905. The repository contains
461 as well the target gene database (probe-capture/2-
462 selected_outputs/all_genes_compiled_ncbi_nt_envnt_fungene_split_file_0.fasta.gz (four files

463 ..file_0-3.fasta.gz), and the final probe sequences (probe-capture/2-
464 selected_outputs/Final_N_probes.list1.fasta).
465
466
467 **Results**
468
469 **Benchmarking gene detection and quantification using targeted metagenomics in mock**
470 **communities**
471 The targeted metagenomic approach was evaluated using mock microbial communities comprising
472 predefined mixtures of genomic DNA from 14 bacterial and three archaeal strains, together
473 containing all 14 functional genes targeted by the probes and mixed in different proportions to
474 produce six different median G+C contents ranging from 47% to 62% (Table S1). The overall
475 relative abundances of target genes in the captured metagenomes across all GC% categories were
476 correlated with their abundance in the original mock communities ($r = 0.78$ ($P < 0.0001$) (Fig. 2).
477 Comparisons between the relative abundance of individual genes in the mock communities as
478 compared to captured metagenomes revealed highly similar values (t = 1.8518e-10, df = 174.83,
479 $P > 0.05$), with the exception of the *nirK* and the *hzoA* genes (Fig. S1a,b). Pearson correlation
480 coefficients across all GC% categories varied between 0.76 and 0.93, although correlations were
481 stronger within higher GC% categories (57%-63%; R = 0.80-0.93) than within lower GC%
482 categories (47%-53%; R = 0.76-0.79) (Fig. S1). The probe capture targeted metagenomics
483 produced 61-72% of target gene sequences from the total sequenced library of the mock-
484 communities (Table S4).
485
486 **Precision of gene identification in mock communities**
487 Precision for the in-house HMMs ranged from 74.1% (*nifH*) to 100% (*mcrA*) (Fig. S5). The
488 average precision of the models was 93.4%, with a median of 99.8% (excluding *amoA* and *pmoA*).
489 The recall ranged from 42.8% (*nxrB*) to 100% (*mcrA*). The average recall was 81.9%, with a
490 median recall of 82.8%. Because KEGG only provides a single model covering the homologous
491 genes *pmoA* and *amoA*, it was not possible to accurately calculate the precision and recall for these
492 genes individually. A precision estimate could still be determined for the two genes, where a "true
493 positive" is defined as a hit for a read identified by the in-house models as *amoA* or *pmoA* mapped

494 to a CDS identified by the HMM for (*a*/*p*)*moA* genes provided by KEGG (K10944). In this case,
495 the precision values for the *amoA* and *pmoA* genes were estimated to be 96.9%, and 100%,
496 respectively. It is important to note that these estimates do not exclude the possibility that the in-
497 house models misclassified reads belonging to these homologous protein families.
498
499 Precision and recall values were similar using KOFAMs instead of in-house HMMs. However, the
500 results for *amoA* and *pmoA* genes were combined due to a single model for the associated KO for
501 both genes, as mentioned above. Precision ranged from 76.5% (*amoA*/*pmoA*) to 100.0% (*mcrA*),
502 while recall varied from 34.3% (*nifH*) to 100% (*mcrA* and *nxrB*). The average and median
503 precision values were 94.5% and 97.7%, while the average and median recall values were 93.39%
504 and 99.03%, respectively. The precision of KOs was lower for *napA* than for other genes when
505 compared with custom DNA HMMs. This could be accounted for by the presence of formate
506 dehydrogenase, a known homolog of *napA*. Formate dehydrogenase (K00123) was indeed
507 identified among the genomes used for the mock community, so it is possible that the custom
508 HMM was misclassifying reads originating from this known ortholog to *napA* (Cerqueira et al.,
509 2015).
510
511 **Higher number of reads for all target functional genes but similar relative abundance**
512 **obtained with targeted compared to shotgun metagenomics from complex environmental**
513 **samples**
514 We investigated the efficiency of the targeted metagenomics approach in natural complex
515 ecosystems by directly comparing this approach with shotgun untargeted metagenomics generated
516 with a nearly 100 times higher amount of sequencing (~66 Mb and ~6.4 Gb per sample,
517 respectively) from wetland soils in Bellefontaine, France, and from an agricultural field in Hungary
518 (Table S4). These soils represent distinct ecosystems with average physicochemical conditions,
519 such as water content and oxygen concentration, and N availability that favors different
520 distributions of functional genes (Jones et al. 2014). Despite the large difference in sequencing
521 depth, targeted metagenomics generated a much larger set of target functional genes (Fig. 3). In
522 both the agricultural and wetland site the targeted metagenomics approach detected 14 out of 15
523 gene-clusters and the shotgun approach 13 out of 15 genes (*mcrA* was not detected in the
524 agricultural site and *hzoA* not in the wetland site). Moreover, up to 60 times as many identified

525 gene sequences were detected using the probe capture compared to shotgun metagenomics (Fig.
526 3, Table S4). The relative abundances of functional genes in captured metagenomes correlated
527 significantly with that in shotgun metagenomes from both the agricultural field (R_{Pearson} = 0.96,
528 d.f.=14, $P < 0.00001$) and the wetland (R_{Pearson} = 0.70, d.f,=14, $P < 0.01$). The relative abundances
529 of target genes were different between the two approaches ($P > 0.00001$) (Fig. 3). Moreover,
530 increased relative abundance was detected with targeted compared to shotgun metagenomics in
531 the agricultural site for *nifH*, *TamoA*, *nrfA*, *napA*, *nirK*, *nirS*, *norB*, *nosZ* and *mmoX* (Fig. 3a) genes
532 and the wetland site for *nifH*, *napA, narG* and *nirS* (Fig. 3b) genes. The targeted approach
533 generated a much higher number of reads for all target genes in both sites than shotgun
534 metagenomics (Target reads in Table S4).
535
536 The different relative abundances of functional genes in the two ecosystems reflected known
537 differences in their environmental conditions: (i) N_2 fixation genes were expectedly higher in the
538 unfertilized wetland; (ii) Genes involved in ammonia oxidation and nitric and nitrous oxide
539 reduction were higher in the agricultural soil, which is likely to be more oxic and also subject to
540 N fertilization which increase inorganic N cycling; (iii) Genes involved in methanogenesis were
541 more abundant in the wetland, where anoxic conditions and methane production typically occur.
542
543 **Comparing diversity recovered for specific target genes from amplicon, targeted and**
544 **shotgun metagenomics from complex environmental samples**
545 In order to compare the diversity of specific functional genes detected by targeted metagenomics,
546 shotgun metagenomics and PCR amplicon sequencing we focused on the archaeal *amoA* gene as
547 a test case, as it is the second most sequenced gene in NCBI indicative of its ecological importance,
548 and for which there is a comprehensive reference database for classification available (Alves et
549 al., 2018). Moreover, archaeal ammonia oxidizers (AOA) typically outnumber their bacterial
550 counterparts in most environments (Leininger et al. 2006), and even though they can be low
551 abundant, their diversity is striking and underlines their distinct metabolic capabilities (Alves et
552 al. 2018; Huang et al. 2021). Additionally, we compared the detection of *nosZ* genes between the
553 targeted and shotgun metagenomics approaches in the agricultural soil, also based on an existing
554 reference database (Graf et al., 2022). The following datasets were compared: (i) targeted and
555 shotgun metagenomes, and PCR amplicons of the archaeal *amoA* gene in the agricultural soil (Fig.

556 4); (ii) *nosZ* gene sequences identified by targeted or shotgun metagenomics in the agricultural
557 soil (Fig. S3).
558
559 Comparison of results from all three methods in agricultural soil samples showed that targeted
560 metagenomics detected a substantially higher diversity of archaeal *amoA* genes than PCR
561 amplicon sequencing, while, as expected, shotgun metagenomics detected a lower diversity than
562 either of the other two methods (Fig. 4A). Specifically, targeted metagenomics detected 71-83
563 distinct taxonomic bins of *amoA* gene, whereas PCR amplicon sequencing and shotgun
564 metagenomics detected 65-68 taxonomic bins and only three bins, respectively. Rarefaction
565 analysis also indicated that targeted metagenomics retrieved a higher alpha diversity of archaeal
566 *amoA* genes than PCR amplicon sequencing (Fig. 4B,C) and it also reaches a plateau much faster
567 and with a lower number of sequences than amplicon sequencing (Fig. 4B).
568
569 Similarly, targeted metagenomes yielded a higher number of taxonomic bins for *nosZ* genes from
570 the agricultural soil (~6,800 bins) than shotgun metagenomics (~1,300 bins) at a 90% identity
571 cutoff, showing that the first approach detected a greater gene diversity with a much lower
572 sequencing depth (Fig. S3). Gene sequence reads from both methods mapped to similar lineages
573 in a comprehensive reference phylogeny of *nosZ* genes (Graf et al., 2022). However, a greater
574 proportion of reads from the targeted metagenomics approach were assigned to deeper nodes of
575 the phylogeny, particularly within the less studied clade II, suggesting that this method also
576 captured more novel *nosZ* variants not represented in the reference phylogeny.
577
578 Targeted metagenomics with a sequencing depth of 117-160 Mb per sample generated at least
579 2200-4200 times more *TamoA* gene reads than shotgun metagenomics with 7-17 Gb per sample
580 (where only three quality-controlled *amoA* taxonomic bins was detected (Fig 4)). Conversely,
581 targeted metagenomics generated five times less *TamoA* reads (Fig. 4B) than PCR amplicon
582 sequencing, with a sequencing depth of 15-31 Mb per sample. Nevertheless, the targeted
583 metagenomics approach detected a much higher gene sequence diversity for both *TamoA* (Fig 4A,
584 4B) and *nosZ* (Fig. S3) than the two other methods in the agricultural soil samples. For *TamoA*,
585 the targeted approach detected 28 times higher alpha-diversity than shotgun metagenomics, while
586 5 times higher diversity of *nosZ* genes was recovered, importantly covering genes affiliated with

587 the less characterized Clade II. In turn, the targeted approach detected 1.24 times (24% more
588 taxonomic bins) higher diversity of archaeal *amoA* genes than PCR amplicon sequencing in
589 agricultural soils, respectively.
590
591 **Discussion**
592 In this study, we report the development and evaluation of a new probe library for targeted
593 metagenomics of 14 key genes involved in the microbial cycling of inorganic N and CH_4. This
594 included the compilation of databases containing all variants of the target genes from public
595 databases, the development of an extensive set of probes targeting 14 well-established marker
596 genes, and a bioinformatics pipeline to process and annotate the sequence data. This approach has
597 been successfully used earlier to identify genes involved in microbial C metabolism in soil
598 (Manoharan et al., 2015, Manoharan et al., 2017).
599
600 Our custom HMM models could assign reads to the correct genomes based on full protein
601 sequences with higher precision than models based on KEGG Orthologs, especially when used to
602 predict putative functions from short DNA fragments, such as metagenomic reads. This accuracy
603 indicates that the HMMs can reliably classify reads in the mock community and are sensitive
604 enough to detect distantly related genes or novel gene variants. Notably, reads identified as false
605 positives may not actually be incorrect but could represent novel gene variants not captured by
606 KEGG Orthologs.
607
608 In validation experiments with mock communities comprising 18 distinct organisms encoding
609 multiple target genes, we showed that this method successfully reproduced the expected relative
610 abundances of nearly all target genes, with the exception of the *nirK* and *hzoA* genes. Although
611 10.3% of the probes targeted *nirK* genes of nitrifiers, these were detected at a lower relative
612 abundance than that present in the original mock community. However, the relative abundance of
613 *nirK* genes matched the frequency of *nirK* genes only in heterotrophic organisms in the mock
614 community, indicating a bias in the detection of distinct *nirK* gene variants. The sequencing
615 technology used can influence the sequences generated due to known GC biases. In this case, the
616 relative abundance of *nirK* genes may have been biased by the false low coverage of genomic
617 regions with non-optimal GC% content (50-60%) known from MiSeq Illumina sequencing

618 (Browne et al. 2020). Consistent with this observation, there was some variation in probe
619 specificity among mock communities with different genomic GC% content, with high GC%
620 communities having a generally higher target gene detection efficiency. For example, the mean
621 GC% content of nitrifier *nirK* genes is lower than that of heterotrophic *nirK* genes (53% and >60%,
622 respectively) and thus it is plausible that the later are sequenced more efficiently. This effect was
623 also noticeable among *nosZ* genes, as the *nosZ* gene of *D. fermentas*, with GC content of 52.9%
624 (Table S1, Fig. S1), was detected more efficiently than that of *S. denitrificans*, which has a 32.7%
625 GC content. Moreover, variation in detection efficiency may also result from differences in probe
626 hybridization. For instance, probes with higher GC% content are expected to bind more strongly
627 to their targets given the higher binding energy between guanine and cytosine than between
628 adenine and thymine. Although our approach successfully identified *hzoA* genes in the
629 environmental samples, it failed to detect them in mock communities. It is possible that this was
630 due to the PCR-generated genomic fragment used as source material for *hzoA* genes in the mock
631 community, which, unlike other target genes provided in the whole genomic DNA, is more
632 susceptible to degradation or structure-related constraints on probe hybridization.
633
634 Targeted metagenomics also closely reproduced the relative abundances of target genes obtained
635 with shotgun metagenomics in two distinct natural microbial communities from soils. The relative
636 gene abundances were strongly positively correlated between the two methods, indicating that the
637 probe capture step did not introduce a significant quantitative bias. Importantly, this relationship
638 held also true for *nirK* genes, confirming that the lower detection efficiency of nitrifier *nirK* genes
639 was due to a variation in GC content. This observation emphasizes that particular attention should
640 be given to the design of probes for low GC genes, such as by targeting gene regions that are not
641 highly conserved among all gene variants, where higher GC probes are more likely to outcompete
642 lower GC probes, as in the case of *nirK*. Nonetheless, as observed in the mock communities, the
643 relative gene abundances quantified in environmental samples were strongly positively correlated
644 between the two approaches to all GC% categories (Fig. S2). This further supports that the
645 observed variation in detection efficiencies among gene variants with targeted metagenomics has
646 a negligible effect on community profiles.
647

648 The thaumarchaeal *amoA* gene (*TamoA*) and the *nosZ* gene were used as case studies to investigate
649 the differences in the detected diversity of single functional guilds that are known to be highly
650 diverse (Alves et al. 2018, Graf et al. 2022). To determine the community composition of the
651 *TamoA* gene for the different sequencing approaches, the reads are mapped to a reference library,
652 in turn forming the taxonomic bins. Consequently, the same gene from the same organism can be
653 detected multiple times in targeted metagenomics due to multiple probe hybridization. However,
654 when calculation of relative abundance is performed, it balances the detected community because
655 an equal number of probes is used per sequence cluster and therefore the relative abundance of
656 targeted method can be compared to other sequencing tools. At first glance, PCR amplicon
657 sequencing seems to have resulted in 18 more taxonomic bins than targeted metagenomics.
658 However, these sequences were generated by a 35-cycle PCR, known to produce some chimeric
659 sequences. Examples of such errors have been shown even for shorter amplification cycles (Kozich
660 et al., 2013). Problems due to chimeric sequences in public databases from amplicon studies are
661 known and were encountered during the compilation of the thaumarchaeal *amoA* reference
662 database (Alves et al., 2018). Although we used standard pipelines for the detection of chimeric
663 sequences, it is possible that we might have missed some, which may be represented in the 18 bins
664 produced with amplicon sequencing, leading to an inflated number of sequences post-processing.
665 The absence of long amplification steps in targeted metagenomics is expected to preclude the
666 formation of chimeric sequences, which represents a significant advantage over typical PCR-based
667 approaches.
668
669 It should be noted that the efficiency of the targeted metagenomic approach largely depends on the
670 coverage and quality of the database used to generate the probes. We used hmmer profiles to
671 collect all possible genes from the NCBI nucleotide and WGS databases, as well as Fungene and
672 other published databases were used for the target gene library. Only one hmmer profile per gene
673 was used for detection, however, clade specific hmmer profiles could also be used to have a
674 broader outcome for neighbouring clades. We used fairly relaxed conditions when including
675 thresholds for screening the NCBI databases, because we wanted to have closely related organisms
676 and genes in the probe pool. For example, this was the case for the *mmoX* gene family, which also
677 includes butane, propane and toluene monooxygenases. We included these closely related *mmoX*
678 genes in the probes, therefore these closely related *mmoX* genes were found from coniferous trees

679 (Putkinen et al., 2021). However, the database is not constrained to full-length gene sequences
680 from a limited diversity of complete genomes and long metagenomic scaffolds available and the
681 probe/target diversity can be greatly extended through the inclusion of gene fragments generated
682 by PCR or from short metagenomic contigs. Despite its high target precision, this method may
683 nevertheless capture non-target sequences, especially for orthologous gene families who have
684 evolved different functions, as is the case for *napA* genes and formate dehydrogenases. Such cases
685 emphasize the importance of thorough data annotation and filtering procedures. With the target
686 gene database used to generate probes in this study, we managed to detect a higher diversity for
687 the case study of *TamoA* genes than the shotgun sequencing approach, showcasing the advantage
688 of "casting a wider net" with targeted metagenomics. Similar results were obtained in other
689 targeted metagenomics applications, such as for detection of diverse resistome-virulome elements
690 (Noyes et al. 2017) or for improved taxonomic microbial community characterization via 16S
691 rRNA enrichment (Beaudry et al. 2021).
692
693 Despite the technological advances and decline in cost of high-throughput sequencing, shotgun
694 metagenomics remain impractical and prohibitively expensive to capture the diversity of specific,
695 low abundant functional groups in complex environments, such as soils and sediments, especially
696 in longitudinal studies. The current cost of generating the probes for targeted metagenomics is
697 about 20-50 € per sample depending on the probe manufacturer. If the goal is to have a focused,
698 comprehensive view of the diversity of functional guilds involved in inorganic nitrogen or methane
699 cycling in a certain ecosystem, then targeted metagenomics can circumvent the high cost and
700 overabundance of data generated by shotgun metagenomics, as well as provide more quantitative
701 data and more information on the diversity of the genes of interest. In turn, PCR-based assays are
702 limited by the biases associated with any set of primers targeting a large diversity of sequences,
703 assay efficiencies which preclude the detection of highly divergent new sequence diversity as well
704 as known issues with the generation of chimeric sequences during amplification. The targeted
705 metagenomics approach circumvents these limitations by using multiple short probes targeting
706 different regions of each gene, which largely increases the likelihood of capturing sequence
707 variants that elude PCR amplification. Thus, this approach has not only the potential to capture
708 rare and novel gene diversity in complex environments, but also to identify cryptic microorganisms
709 in low-biomass samples or involved in suggested CH_4 metabolisms, such as in the tree

710 phyllosphere (Putkinen et al., 2021) and nitrogen cycling in coral holobiont (Glaze et al., 2021).
711 Moreover, targeted metagenomics can also overcome issues associated with running and
712 comparing multiple independent PCR assays when investigating several distinct targets. In that
713 sense, this approach effectively represents a PCR-independent, multiplex approach to characterize
714 simultaneously and in-depth the distributions of a broad range of functional genes, providing a
715 holistic view of the status of the nitrogen and methane cycles in the studied ecosystems. This is
716 especially advantageous when combined with functional studies, such as the determination of N-
717 transformation rates and *in situ* fluxes, as showcased by a study of N_2O emissions in thawing
718 Yedoma permafrost sites over time (Maruschak et al. 2021). In this study, the application of
719 targeted metagenomics with the N-cycling probe dataset presented here revealed that changes in
720 the N-cycling microbial community composition were responsible for an increase in N_2O
721 emissions in revegetated Yedoma soils, which had undergone thawing a decade prior.
722
723 Conclusively, the targeted metagenomics approach developed here provides an efficient and cost-
724 effective strategy for studying microbial functional guilds that typically represent small fractions
725 of natural microbiomes, and whose diversity is generally underestimated and highly
726 underrepresented in metagenomic datasets. This approach also circumvents the limitations and
727 biases associated with PCR-based methods and has higher potential to capture rare or novel
728 functional gene diversity.
729
730
731
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741

742 **Authors contribution statement**

743 H.S., C.S., R.A. designed study, H.S. collected the targeted gene library, R.A.,S.H.,C.J. provided
744 reference libraries for *amoA* and *nosZ* genes, L.M. designed the probes, L.M., A.B., H.S. extracted
745 the environmental DNA and did the shotgun metagenomics, H.S. and S.H. extracted and provided
746 the mock sample cultured organisms, A.H., F.S. did the precision and recall analysis between the
747 KEGG and HMM approaches, A.B., H.S. did the *amoA* diversity comparison, S.H.,C.J. did the
748 *nosZ* diversity comparison. H.S. wrote the first version of manuscript and all co-authors further
749 developed the text.

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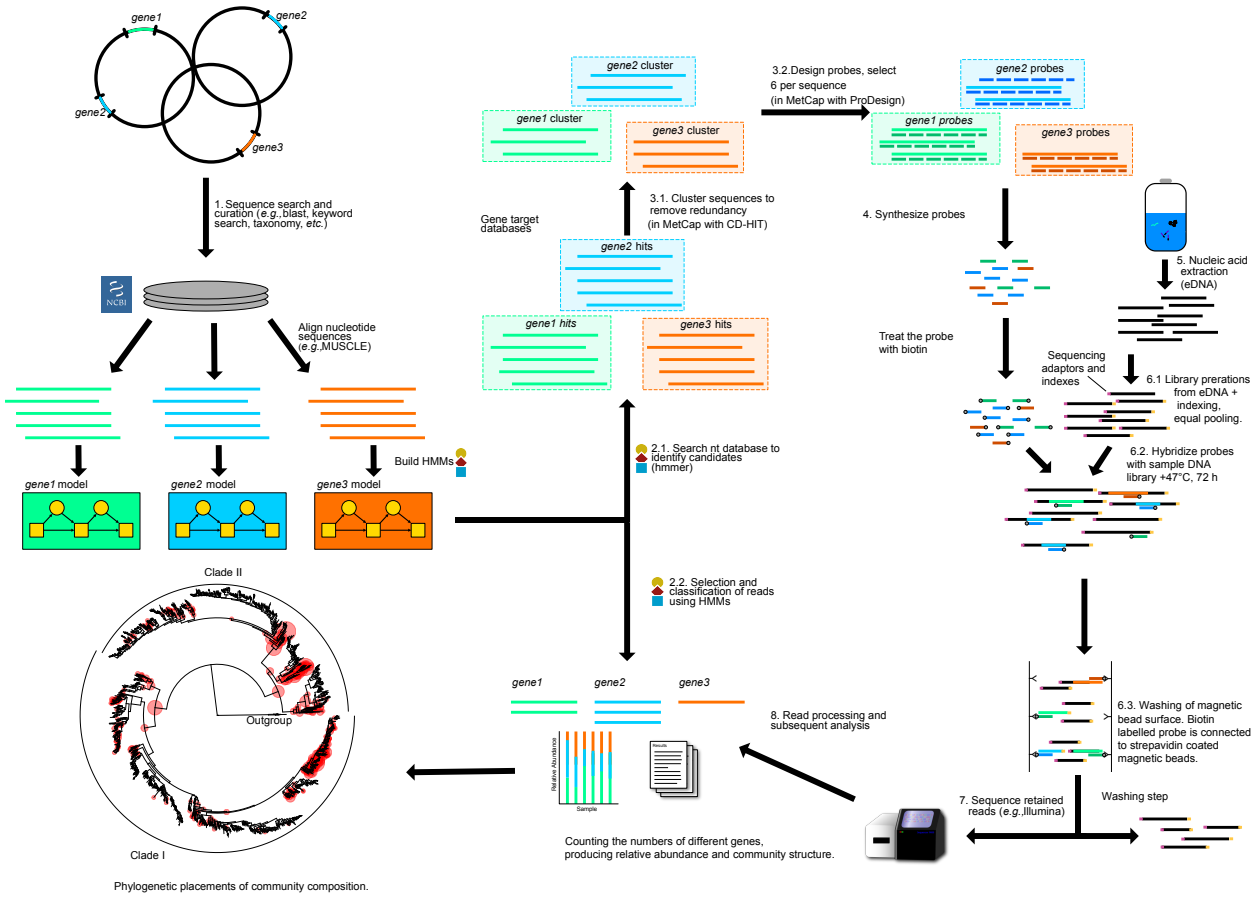
1099 **Figures:**



1100

1101 Fig. 1 Illustration of the pipeline for the generation of HMM models for each gene, and how these
1102 models are used to study community composition with targeted metagenomics: 1. Steps followed
1103 for the generation of sequence databases for the genes of interest and subsequent building of the
1104 respective HMM models for each gene; 2.1. The produced HMM models were used to recruit more
1105 gene variants from the NCBI nt database and generate a target gene database for probe design; 2.2.
1106 The same HMM models were also used to classify the reads captured downstream; 3.1. Clustering
1107 of the target gene database with CD-HIT (in MetCap pipeline); 3.2. Design and selection of six
1108 50-mer probes for each sequence cluster (in MetCap pipeline); 4. Synthesis and biotinylation of
1109 the designed probes by Roche; 5. DNA extraction of the sample of interest; 6.1. Sequencing
1110 libraries preparation and addition of sequencing adaptors and indexes; 6.2. Hybridization of the
1111 probes to indexed DNA libraries for 72 hours at +47°C; 6.3. Purification of the DNA hybridized
1112 to the probes with streptavidin coated magnetic beads; 7. Sequencing of the hybridized libraries

1113 with Illumina MiSeq; 8. Read processing with HMM profiles and taxonomy assignment for the
1114 genes of interest.
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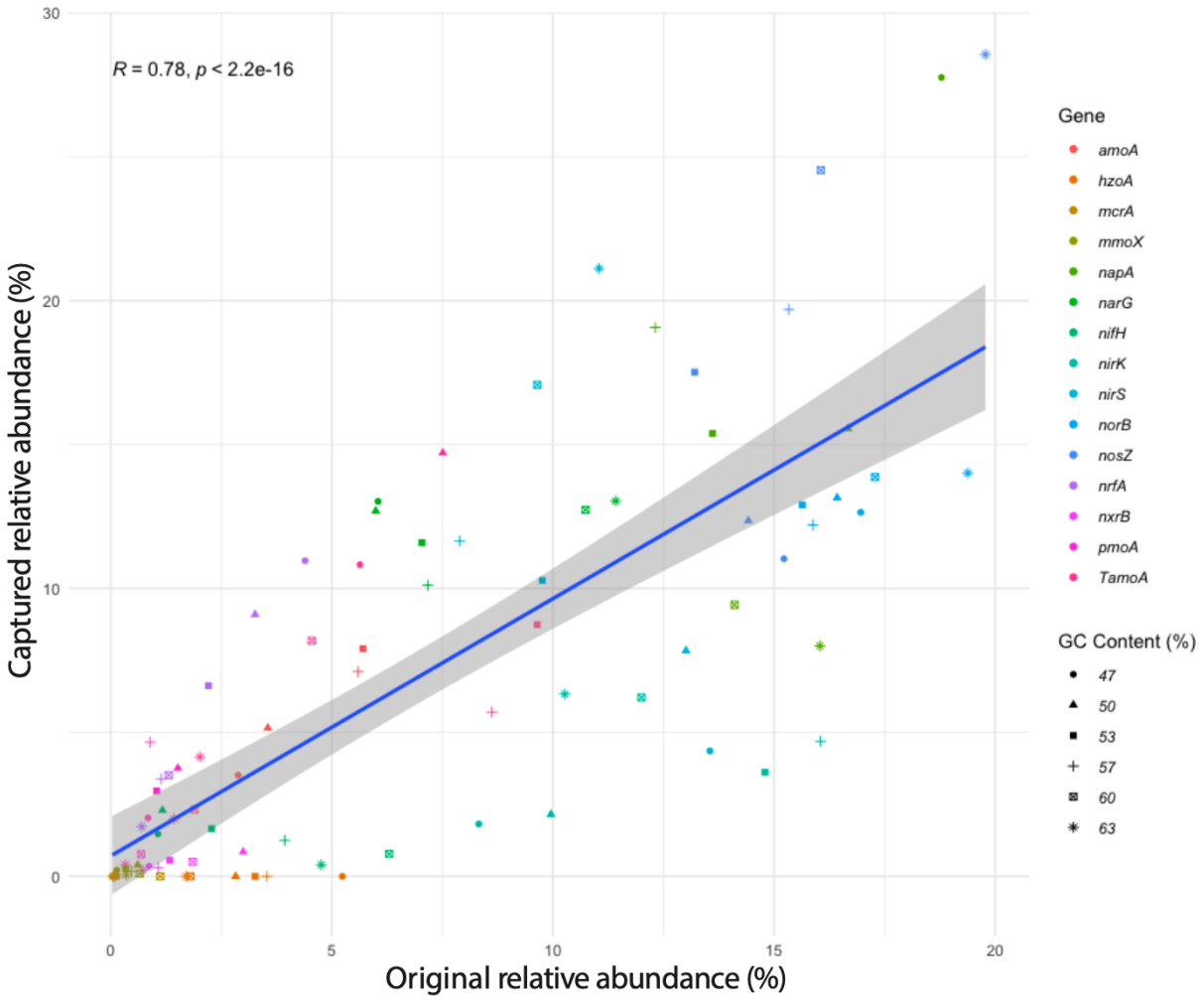
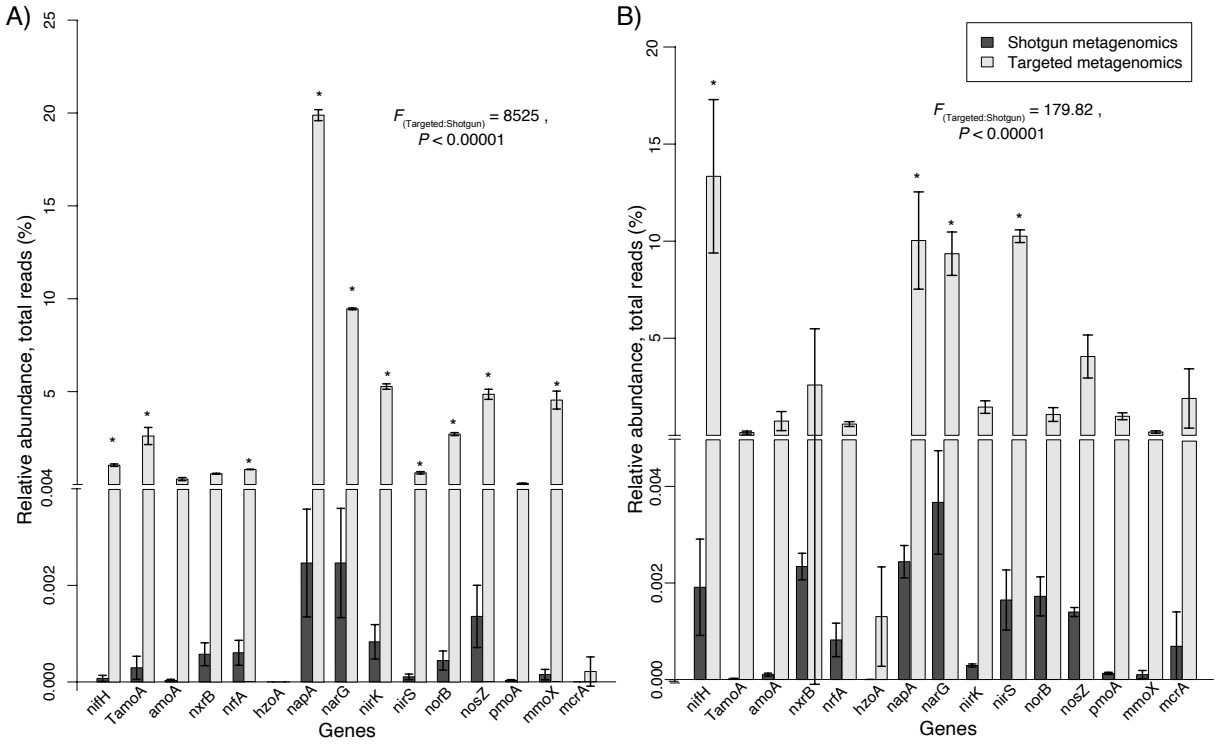
1116



1117
1118
1119 Fig. 2. Comparison between the original composition (DNA of strains applied into the mock
1120 sample) of the mock community and the relative abundance for each functional gene detected
1121 using probe captured metagenomics for all GC% combinations combined. The relative abundance
1122 in the mock community was calculated based on the amount of each functional gene and organism
1123 as well as genome size. Color of the symbols depict gene and shape depicts the category of GC
1124 content (%).
1125

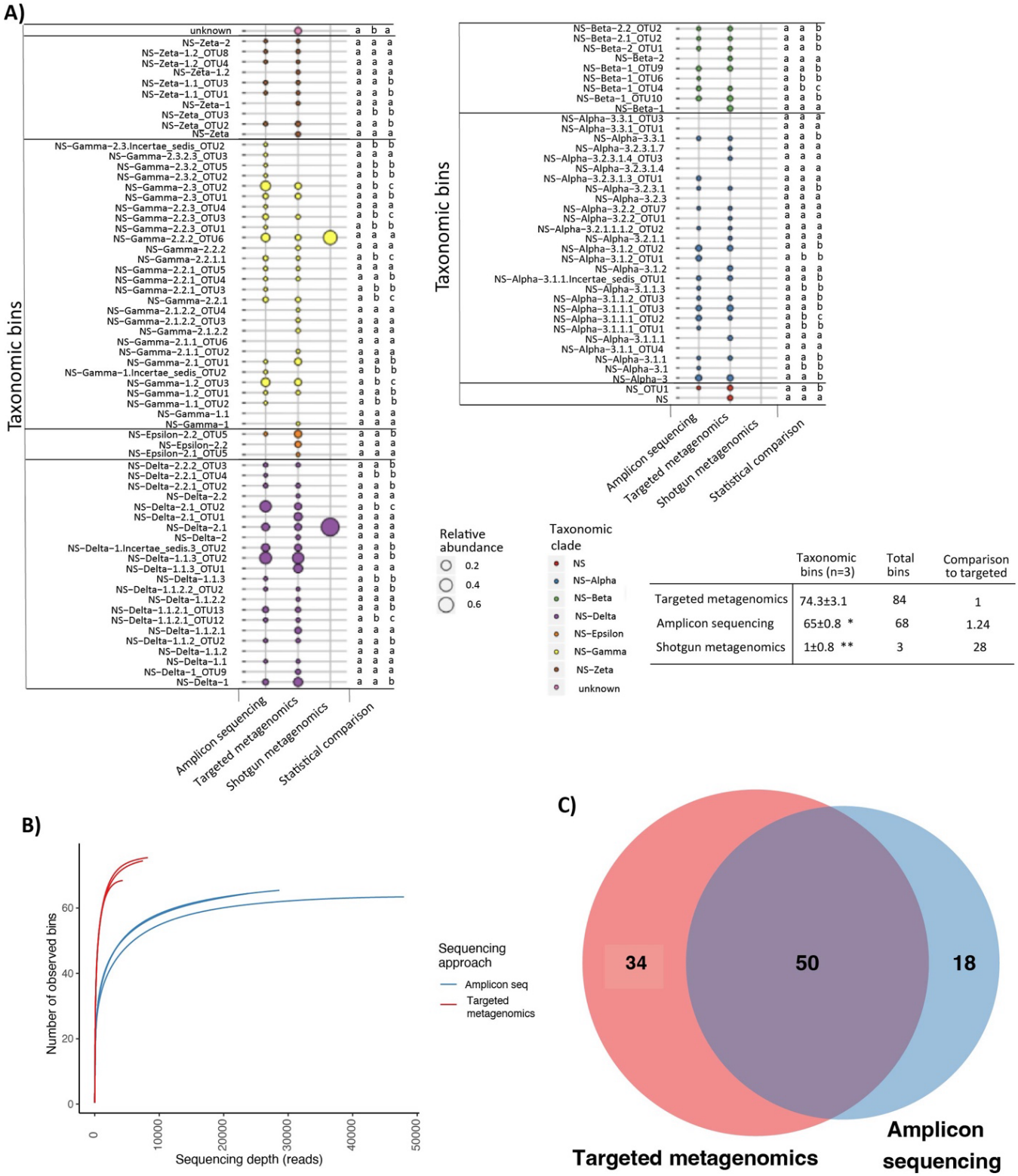
1126



1127

1128 Fig. 3. The relative abundance of functional genes (calculated against total sequencing reads)
1129 involved in inorganic nitrogen and methane cycling obtained by targeted metagenomics and
1130 shotgun metagenomics from A) agricultural soil; and B) wetland soil. Relative abundance was
1131 retrieved with HMMER searches of each functional gene. Statistically significant differences
1132 between shotgun and targeted relative abundances according to pairwise comparisons with
1133 ANOVA are shown with asterisk (P < 0.05, n = 3). The comparison of shotgun vs. targeted
1134 metagenomic measured with ANOVA is shown as F values and significance in the inset for both
1135 samples.

1136



	Taxonomic bins (n=3)	Total bins	Comparison to targeted
Targeted metagenomics	74.3±3.1	84	1
Amplicon sequencing	65±0.8 *	68	1.24
Shotgun metagenomics	1±0.8 **	3	28

1141
1142 Fig. 4. A) The relative abundance of archaeal *amoA* taxonomic bins produced from amplicon
1143 sequencing with *amoA* amplicon sequencing (*amoA* gene PCR amplicons sequenced with Illumina
1144 MiSeq, about 22000-47000 *amoA* reads per sample) (n = 3), metagenomics reads of targeted
1145 metagenomics (genomic DNA sequenced with Illumina MiSeq, after target enrichment, about
1146 4500-8500 *amoA* reads out of 220000-310000 targeted reads per sample)(n = 3), or shotgun
1147 metagenomics (genomic DNA sequenced with Illumina HiSeq, about 22-57 million reads per
1148 sample, of which only two were archaeal *amoA*)(n = 3). In all cases, the analysis pipeline consisted
1149 of USEARCH comparison on *amoA* reference database (Alves et al., 2018) and taxonomy were
1150 analyzed with QIIME1 analytical pipeline. Sample having largest diversity is shown (n = 3).
1151 Bubble symbol size is proportional to the relative abundance of each taxonomic bin produced with
1152 sequencing approach in each sequencing method. Color indicates Nitrosospheria (NS)-clade of
1153 taxonomic bin. Statistically significant differences in abundance of each taxonomic bins between
1154 sequencing approaches based on Pairwise-Wilcox comparison are indicated with different letters
1155 ($P < 0.05$, n=3), and the significant difference of comparison for taxonomic bins is shown an
1156 asterisk, * $P<0.05$, ** $P<0.01$. B) The rarefaction analysis of archaeal *amoA* obtained by amplicon
1157 sequencing and captured metagenomics. C) Venn-diagram of archaeal *amoA* sequences obtained
1158 by amplicon sequencing and targeted metagenomics.
1159

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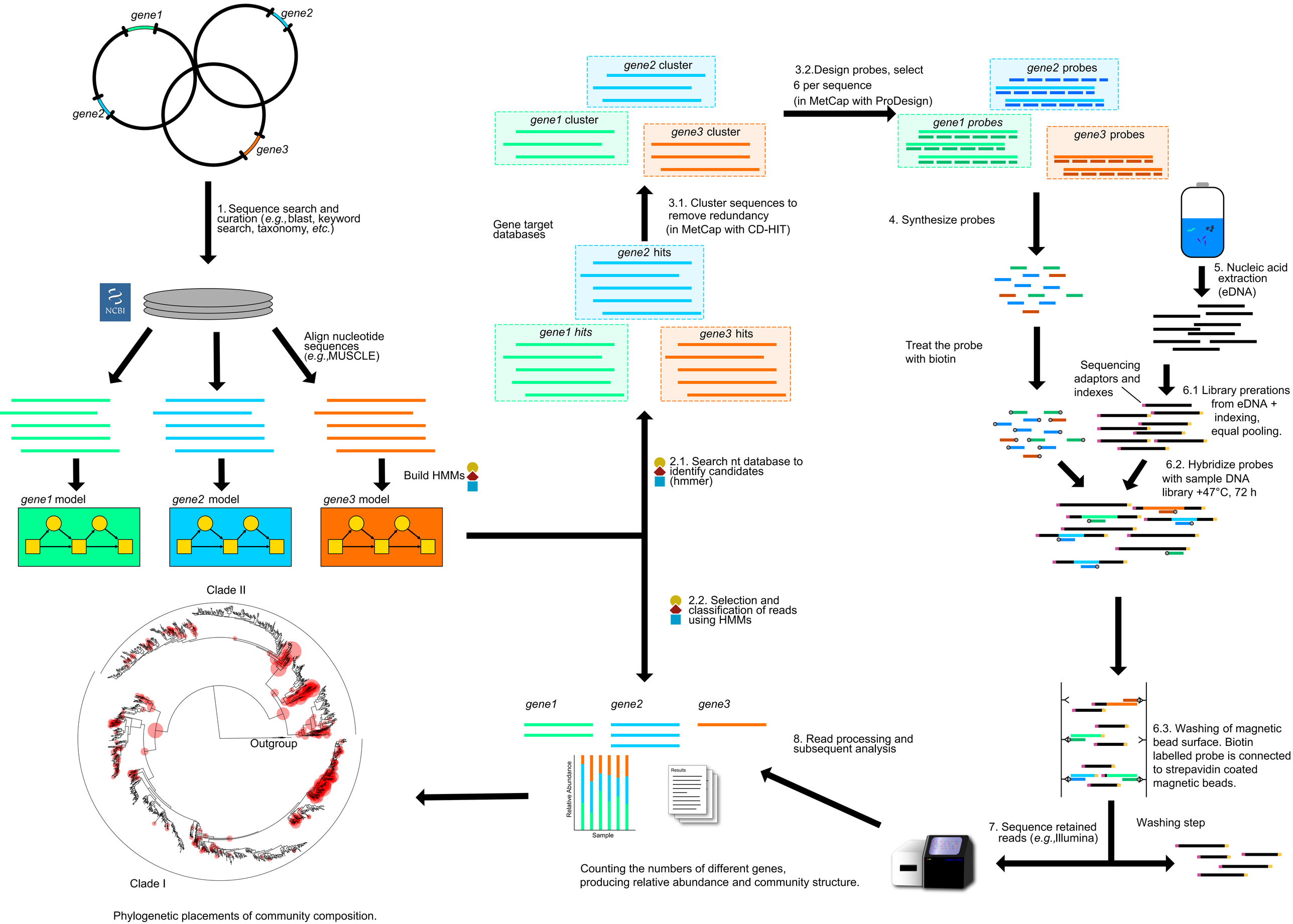
gene1
gene2
gene2
gene3
1. Sequence search and curation (e.g., blast, keyword search, taxonomy, etc.)
NCBI
Align nucleotide sequences (e.g.,MUSCLE)
gene1 model
gene2 model
gene3 model
Build HMMs
Clade II
Outgroup
Clade I
Phylogenetic placements of community composition.
Gene target databases
gene2 cluster
gene1 cluster
gene3 cluster
3.1. Cluster sequences to remove redundancy (in MetCap with CD-HIT)
gene2 hits
gene1 hits
gene3 hits
2.1. Search nt database to identify candidates (hmmer)
2.2. Selection and classification of reads using HMMs
gene1
gene2
gene3
Relative Abundance
Sample
Results
Counting the numbers of different genes, producing relative abundance and community structure.
8. Read processing and subsequent analysis
3.2.Design probes, select 6 per sequence (in MetCap with ProDesign)
gene2 probes
gene1 probes
gene3 probes
4. Synthesize probes
Treat the probe with biotin
5. Nucleic acid extraction (eDNA)
Sequencing adaptors and indexes
6.1 Library prerations from eDNA + indexing, equal pooling.
6.2. Hybridize probes with sample DNA library +47°C, 72 h
6.3. Washing of magnetic bead surface. Biotin labelled probe is connected to strepavidin coated magnetic beads.
Washing step
7. Sequence retained reads (e.g.,Illumina)