LOW NITROGEN (N) use efficiency in agriculture is a global issue, as roughly half of all N added to soils is not incorporated by crops and is lost to the environment (Galloway et al., 2014). Inasmuch as human decision-making drives N fertilizer consumption, our understanding of how to reduce N loss from soils is hampered by an inadequate grasp of microbial processes affecting N retention and mobility. High-throughput sequencing (HTS) of microbial DNA is enabling fundamental insights into N cycling microorganisms and their metabolisms. This commentary describes six emerging technologies that could be combined with HTS to enable real-time collection of metadata on N transformations, intermediates, and products to link soil properties, microbial processes, and the fate of N. These technologies include microdialysis and microfluidics, automated sensing, microfabrication of model soils, parallel quantification of N functional genes, sorting active cells, and stable isotope probing. Use of integrated technologies applied initially under controlled conditions at small scales can lead to identification of soil conditions and field-scale management practices that promote better N conservation and delivery to agricultural crops.

Innovative Technologies Can Improve Understanding of Microbial Nitrogen Dynamics in Agricultural Soils

Mara Lee Cloutier, Arnab Bhowmik, Terrence H. Bell, and Mary Ann Bruns*

Abstract: Roughly half of all nitrogen (N) added to soils is not incorporated by crops and is lost to the environment (Galloway et al., 2014). Inasmuch as human decision-making drives N fertilizer consumption, our understanding of how to reduce N loss from soils is hampered by our inadequate grasp of the microbial processes affecting N retention and mobility (Kuypers et al., 2018). Predicting N fate in agroecosystems remains elusive since microbial N cycling comprises multiple organic and inorganic forms of N that are produced, altered, and assimilated by diverse microbial taxa. Measurement of soil N in situ, often as nitrate (NO$_3^-$-N) at a single time point, is further complicated by the various forms of N-containing organic compounds with different susceptibilities to mineralization, the concentrations of which can change seasonally and depend on the type of crop grown and type of fertilizer used (i.e., synthetic, animal or green manure, or compost).

A simplified diagram showing microbially mediated N cycle transformations (Fig. 1) reflects the metabolic versatility of microbes that may use N either as a nutrient for assimilation and biosynthesis, as an electron donor for energy, or as an electron acceptor during respiration (Butterbach-Bahl et al., 2013). Microbial activities that modify N oxidation states strongly influence N residence times in soils. Ammonium (NH$_4^+$, the least-oxidized N form) is held by soil cation exchange sites, while the most oxidized forms (nitrates, or NO$_3^-$) are mobile and readily leached. Retention time can also be affected by the size and activity of the soil microbial biomass, which drives cyclic microbial N assimilation, storage, degradation, and release before N is taken up by crops. In Fig. 1, N-conserving processes are represented by biological N$_2$ fixation (labeled 1 in Fig. 1), microbial assimilation of inorganic N (2), remineralization (ammonification) of organic N (3), and dissimilatory nitrate reduction to ammonium (DNRA) (4). The N-mobilizing processes of nitrification (5) and denitrification

Abbreviations: BONCAT, biorthogonal noncanonical amino-acid tagging; DNRA, dissimilatory nitrate reduction to ammonium; HTS, high-throughput sequencing; NiCE, nitrogen cycle evaluation; PCR, polymerase chain reaction; qPCR, quantitative polymerase chain reaction; SIP, stable isotope probing.
(7) promote N loss by leaching and gas transfers to the atmosphere, although nitrates from nitrification can also be taken up by crops or microbes and reduced for incorporation into cellular N. Much less is known about the occurrence in soils of anaerobic ammonia oxidation, or anammox (6), and non-denitrifier nitrous oxide (N₂O) reduction (8), which likely requires study at very fine spatial scales. More efficacious N delivery to crops calls for understanding soil conditions that promote N-retaining microbial processes when crop uptake is minimal and N-mobilizing reactions when crop demand is high (Jones et al., 2014; Bhowmik et al., 2017).

We highlight here six emerging technologies that could be combined with high-throughput sequencing (HTS) of DNA to determine soil conditions and microbial functions that foster N conservation. Table 1 describes these technologies, their advantages and disadvantages and identifies some hypotheses related to N-cycling processes (as numbered in Fig. 1) that could be tested using combined technologies. We describe how integrating these methods with HTS could assist in identifying responsible taxa, their enzyme functions, and metabolite transfers that correlate with environmental variables at appropriate scales. Ideally, initial experimentation should be done under controlled conditions, using highly resolved measurements with isotopic tracers for assessing gross, rather than net, N transformations (Wen et al., 2016). Ultimately, this information would need to be scaled to field level to evaluate how N-conserving soil conditions can be achieved through agricultural management.

**Emerging Technologies for Integration with High-Throughput Sequencing**

**Improved Tracking of Nitrogen Cycling Intermediates and Products**

Standard agronomic measurements of soil inorganic N from bulk extracts of composited field samples are insufficient for capturing spatial patterns of N mineralization, nitrification, and denitrification, which often result in “hot spots” or “hot moments” of N loss (see review by Groffman, 2012) (Fig. 1). Estimates of nitrate, assumed to be the predominant form of inorganic N in agricultural soils, are typically used to calculate pre-side dress or split N fertilizer applications. Nitrite, in contrast, is rarely measured because it is highly reactive and often not detected at all in field studies, even though transient, elevated concentrations of nitrite may be critical in determining overall N fate (Maharjan and Venterea, 2013). Capturing the dynamics of nitrite production and transformation in soil may therefore require better-resolved studies at suitable spatial and temporal scales.

**Microdialysis and Microfluidics**

Microdialysis enables fine-scale extraction of solutions from undisturbed soils using capillary-sized probes connected to inlet and outlet tubing. Depending on concentration, aqueous solutions flowing at controlled rates can permit the introduction or uptake of solutes through semipermeable membranes located at the tips or along the inserted probes. Soil microdialysis studies have included measurements of inorganic N (Buckley et al., 2017) and amino acids (Hill et al., 2019), as well as N₂O and NO measurements (Leitner et al., 2017). Identification of both organic and inorganic forms of N in soil solutions using microdialysis (Inselsbacher et al., 2011) coupled with HTS could provide insights on remineralization of specific compounds by particular taxa and identification of conditions that affect ammonium availability. Recently, Warren (2018) coupled microdialysis of soil solution samples with mass spectrometry to measure changes in amino acid concentrations to assess differential microbial uptake. Apart from microdialysis, microfluidics can control mass flows through constrained volumes of soil (Alekklet et al., 2018) to synchronize substrate introductions or metabolite removals with HTS-based transcriptomics and proteomics.

**Automated Sensing**

Automated sensing technologies (Shade et al., 2009) are being explored to assess fine-scale changes in moisture, pH, and redox and changes to N speciation in situ (Garland et al., 2018; Jenkins et al., 2019). Increased frequency of analyte measurement enabled by automated sensors can be coupled with HTS-enabled gene expression to identify active taxa. Several innovative sensing technologies have recently been used for nitrate quantification in soil: microfluidics coupled with electrophoretic microchips that do not require soil solution extraction (Xu et al., 2017); Fourier transform infrared spectroscopy (Rogovska et al., 2019); and laser-induced graphene electrodes capable of recovering more than 95% of nitrate and ammonium added to soil slurries (Garland et al., 2018). Although studies describing these three sensing technologies used point measurements, these sensors could be used with microfluidics adapted for continuous or semi-continuous monitoring of multiple N species.

**Improved Control of Microbial Habitat Conditions**

**Microfabrication of Model Soils**

Difficulties in linking soil conditions with N fate arise because of incongruencies between the scales at which physicochemical parameters are measured and the microsites supporting microbial processes. Construction of artificial

![Fig. 1. Microbially mediated steps in the N cycle. Numbers associated with individual reactions correspond to numbers from Table 1 under "N cycle reactions."](Image)
environments that simulate soil structure (e.g., pores and aggregates) can be achieved through engineering microstructured chips via silicone molding or three-dimensional printing (Aleklett et al., 2018). Microscale habitat design could incorporate differences in porosities and hydraulic conductivities. Integration of microfluidics can better mimic soil solution chemistry through creation of gradients in moisture, N species, carbon (C) substrates, redox, or a combination of these parameters (Aleklett et al., 2018; Stanley and van der Heijden, 2017). Chemical exchanges between microbes and plant roots also can be measured using ecosystem fabrication devices (“EcoFABs”) produced by three-dimensional printing and combined with microfluidics (Gao et al., 2018). Ecosystem fabrication devices containing *Brachypodium distachyon* (L.) P. Beauv. seedlings were used to measure root growth and metabolite production using liquid chromatography–mass spectrometry. When *B. distachyon* was grown in soil extract, it produced more root hairs and exuded less metabolites than when it was grown in synthetic media (Sasse et al., 2019). These tools could thus reveal synchronies between plant exudation and microbial assimilation or mineralization of N.

Table 1. Description and details for methods that can be combined with high-throughput sequencing (HTS) to further our understanding of microbial N cycling in agroecosystems. Numbers associated with N cycle reactions correspond to the numbers in Fig. 1.

<table>
<thead>
<tr>
<th>Method</th>
<th>Description</th>
<th>Pros</th>
<th>Cons</th>
<th>N cycle reactions</th>
</tr>
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<tbody>
<tr>
<td><strong>Microdialysis, microfluidics, automated sensing</strong></td>
<td>Measurements made with microdialysis probes inserted into soil or with mass microfluidic flow through specific volumes of fabricated soils. Fluids can be spiked with internal standards to improve signal detection. Unknown compounds can be identified and quantified using electrospray ionization mass spectrometry.</td>
<td>Increased frequency of measurements in real time and at scales relevant for tracking changes in concentrations of introduced substrates, products, and intermediates. May require extensive trial and error to identify appropriate concentrations and reaction times. Detection sensitivity may be insufficient.</td>
<td>2, 3, 5, 6</td>
<td></td>
</tr>
<tr>
<td><strong>Microfabrication of model soils</strong></td>
<td>Microchannels are etched onto a material, molded using silicone rubber, or printed three dimensionally and connected to input and output tubing for introduction and collection of gases or solutions. Multiple input solutions or gases can be used to create chemical gradients. Can create two- or three-dimensional constructs with different pore sizes, pore densities, hydraulic conductivities, attachment surfaces. Highly customizable. Printed or etched constructs cannot mimic naturally formed soils that have highly heterogeneous organic and mineral compositions.</td>
<td></td>
<td>1, 3, 4, 5, 6, 7, 8</td>
<td></td>
</tr>
<tr>
<td><strong>N cycle evaluation (NiCE) Chip</strong></td>
<td>DNA extracts are loaded into reaction chambers of a Fluidigm Dynamic Array, with amplification reagents assigned through integrated circuitry. <em>Array</em> is read with instrument acting as both thermal-cycler and imager for quantification. Amplified genes can be tagged with adapters for high-throughput amplicon sequencing. Multiplexed amplification reduces cost and time compared with standard quantitative polymerase chain reaction. Amplicons can be subjected to HTS for phylogenetic identification. Requires polymerase chain reaction amplification of genes and its potential bias. Troubleshooting may be needed to decrease nonspecific amplification.</td>
<td></td>
<td>1, 3, 4, 5, 6, 7, 8</td>
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<tr>
<td><strong>NanoString</strong></td>
<td>Multiple DNA or mRNA targets can be detected without polymerase chain reaction using a capture probe and fluorescently labeled reporter probe designed for each target sequence. Targets are hybridized with capture probes that form complexes for immobilization and separation from noncomplexed molecules, and fluorescence is quantified by a digital analyzer. Does not require gene amplification. There are specific probes for each DNA or mRNA target. Can target low-abundance mRNA. Integration of NanoString with HTS permits phylogenetic identification of mRNA and helps link specific organisms to N-cycling reactions. Too specific to discover unidentified genes or gene clades; may require too many unique probes for less-conserved genes.</td>
<td></td>
<td>1, 3, 4, 5, 6, 7, 8</td>
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<tr>
<td><strong>Stable isotope probing (SIP)</strong></td>
<td>Cellular uptake of stable isotope-labeled compounds permits separation from nonlabeled fractions. Samples for SIP-DNA are sequenced on a HTS platform, while proteins are subjected to mass spectrometry (SIP-Protein). Integration of SIP with HTS will allow for the identification of the taxa that are growing or producing the N-cycling proteins in response to the experimental treatments. Cross-feeding is difficult to decouple; protein identification may not be able to distinguish different isoenzymes.</td>
<td></td>
<td>1, 2, 4, 5, 6, 7, 8</td>
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<tr>
<td><strong>Biorthogonal noncanonical amino-acid tagging (BONCAT)</strong></td>
<td>Soils are incubated with a methionine analog, L-homopropargylglycine, which is taken up by active cells for subsequent “click” chemical reactions for fluorescence detection by microscopy or flow cytometry. Sorted cells can be prepared for amplicon sequencing. By sorting active and nonactive fractions of microorganisms, HTS permits phylogenetic identification and expressed gene functions. Proteomics could identify newly synthesized proteins (e.g., transcription factors in specific regulatory pathways). Nonuniform distribution of analog and click reactants and therefore nonuniform cellular uptake. Potential competitive interference by native methionine.</td>
<td></td>
<td>1, 2, 3, 4, 5, 6, 7, 8</td>
<td></td>
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</table>
Parallel Quantification of Functional Genes

Nitrogen Cycle Evaluation (NiCE) Chip

Functional genes directly involved in N cycling represent less than 1% of genes from soil metagenomes (Nelson et al., 2016). Thus, techniques that target specific N-cycling genes would enable identification and quantification of these genes at reduced cost. The N cycle evaluation (NiCE) chip is a microfluidic quantitative polymerase chain reaction (qPCR) micro-array for high-throughput assessment of functional genes in the N cycle (Spurgeon et al., 2008; Oshiki et al., 2018). Multiplex ability to capture gene abundances decreases the cost and time relative to standard qPCR (Oshiki et al., 2018). Moreover, amplified genes on the NiCE Chip can be tagged with adapters and sequenced on an HTS platform. This permits massively parallel quantification via NiCE coupled with phylogenetic and diversity information through HTS of multiple N-cycling genes, thus addressing the need for intensified spatial and temporal analyses of many samples.

NanoString

Although the NiCE chip is a highly parallel technology, it still requires polymerase chain reaction (PCR) amplification that can bias results. NanoString circumvents the need for amplification, increases the sensitivity of quantifying and annotating mRNA transcripts, and eliminates PCR biases (Geiss et al., 2008). NanoString requires development of probes that hybridize with each DNA or mRNA of interest, resulting in its direct quantification (Geiss et al., 2008). The specificity of mRNA probes for the N cycle would overcome the need for more costly metagenomic or metatranscriptomic sequencing. That is not to say, however, that NanoString should not be combined with HTS. Combinations linking HTS adaptors to mRNA molecules after NanoString quantification could provide information regarding the identity and diversity of actively transcribing organisms (Karlsson and Staaf, 2019). Collectively, mRNA quantification via NanoString combined with phylogenetic identification from HTS can allow for in-depth analysis of N cycle enzymes and increase our ability to link active N cycling taxa with soil conditions.

Characterizing Active Microbes and Identifying Newly Synthesized Proteins

Stable Isotope Probing

Microbial cells’ assimilation of compounds labeled with stable isotopes of contrasting atomic weights enables gravimetric or spectrometric separation from cells that have not taken up the labeled compounds. Metagenomic or metatranscriptomic sequencing of stable isotope probing (SIP)–labeled fractions of the microbial community can identify members involved in active metabolism (Coyotzi et al., 2017). In addition, SIP can be used to identify newly synthesized proteins in soil (Starke et al., 2016) or estimate growth rates of bacteria (Li et al., 2019). Active denitrifiers from agricultural soils amended with glucose were identified in a study using SIP with flow-through reactors and 16S rRNA gene amplicon and metagenomic sequencing (Coyotzi et al., 2017). Results indicated that nirK denitrifiers (mostly Betaproteobacteria) were the most active nitrite reducers following glucose additions, and assemblages of bacteria with N₂O reductase (nosZ) genes were dominated by different clades pre- and postincubation. This led to the hypothesis that rare denitrifiers, common to many environments, can be stimulated by environmental cues and may play a significant role in regulating soil N cycling (Coyotzi et al., 2017).

A limitation of using isotope labeling of DNA to study the cascade of transformations in the N cycle is that DNA synthesis is only indirectly linked to denitrification, a respiratory rather than an assimilatory activity (Coyotzi et al., 2017). Since SIP requires addition of labeled substrates that are assimilated into biomass, the ¹⁵N will be incorporated by many actively growing organisms, and not specifically those that transform N (Bell et al., 2011). Proteomics coupled with SIP also could be used to determine which compounds are being assimilated. An approach used by Starke et al. (2016) involved incubation of soil with ¹⁵N- and ¹⁴N-labeled litter to assess the relationship between bacterial and fungal assimilation of plant-derived N. Integrating metaproteomics with SIP and HTS led these authors to determine that Rhizobiales and Actinomycetales were the fastest to respond and assimilate ¹⁵N, while fungi were slower (Starke et al., 2016). This synergistic technique could be applied to soils under different management regimes to determine their impacts on bacterial and fungal N assimilation; or competitive dynamics between nirK- and nirS-containing denitrifiers.

Protein-SIP coupled with HTS may also help resolve the question of how soil oxygen affects the functionality of N₂O reductases that regulate N₂O production from soil systems (Arnosti et al., 2014; Hallin et al., 2018). Such reductases are sensitive to oxygen (Giles et al., 2012), but recent research suggests that Nos can be functional under microaerophilic conditions. Suenaga et al. (2018) demonstrated that Nos functionality in the presence of oxygen was strain-dependent. Two strains possessing the nosZI gene could reduce N₂O in the presence of oxygen, while two other strains having the nosZI gene did not produce active Nos enzymes. Coupling of nitrification-DNRA–denitrification reactions could also be investigated in this way.

Tagging Cells with Noncanonical Amino Acids

Large proportions of soil microbial communities are dormant or inactive, which interferes with HTS analyses. Metabolically active and inactive cells can be distinguished with the use of biorthogonal noncanonical amino-acid tagging (BONCAT) combined with fluorescent labels that undergo “click” reactions with the analog. Labeled cells can be detected with fluorescent in situ hybridization (FISH) microscopy (Hatzenpichler et al., 2014, 2016). Bulk cell mixtures may also be separated into active and less-active fractions by fluorescence-assisted cell sorting with flow cytometry (Couradeau et al., 2018). This technique has been used to characterize spatial distribution of antibiotic resistant cells in biofilms (Babin et al., 2017) and to identify
changes in protein synthesis within a consortium responsible for anaerobic oxidation of methane following the addition of methane (Hatzenpichler et al., 2016). While reverse transcription PCR has largely been used to measure specific gene expression, BONCAT is capable of labeling newly synthesized N-cycle proteins, which can subsequently be identified with proteomic techniques (Hatzenpichler et al., 2014). Integration of HTS, BONCAT, and mineralization or denitrification assays could help differentiate microbial DNA and/or denitrification dynamics in soils under diverse management strategies.

**Future Directions for High-Throughput Sequencing Synergies**

We have highlighted potential synergies between HTS and several innovative technologies and described how they might be combined to elucidate microbial interactions and processes that affect N fate in soils. Controlled incubation experiments at small scales can improve our understanding of environmental variables influencing activities of N-cycling taxa and their responses to fertilization, carbon substrates, and community diversity (Hartman et al., 2017). Such integrative studies can eventually inform higher-level, multispecies metabolic modeling efforts (Henry et al., 2016), which take advantage of a growing and accessible knowledge base of microbial and plant genomes (Arkin et al., 2018). Combining emerging technologies with HTS at small scales could thus yield fundamental insights for developing “target” soil conditions at field scales to foster soil consortia that recycle and deliver N efficiently to agricultural crops. Prospects for scaling up outcomes of processes under highly controlled conditions, however, will be clearly challenged by the tremendous spatial and temporal variability of real-world agricultural fields. Therefore, applying insights from small-scale, laboratory-based experiments to the development of integrative management systems for field-level measurements and evaluative modeling will be needed to further our understanding of N-cycle dynamics in agroecosystems.

**Conflict of Interest**

The authors declare no conflict of interest.

**Acknowledgments**

The authors would like to acknowledge the helpful comments of anonymous reviewers on an earlier manuscript version. Preparation of this commentary was supported by USDA-NIFA Hatch project 1003466, USDA NESARE Graduate Student Research grant GNE18-168 to M.L.C., and USDA NIFA grant 2016-67003-24966 to M.A.B.

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