# Anthropogenic N – A global issue examined at regional scale from soils, to fungi, roots and tree rings

*Martine M.* Savard[1,*], *Christian* Bégin[1], *Jérôme* Laganière[2], *Christine* Martineau[2], *Joëlle* Marion[1], *Franck O.P.* Stefani[2], *Armand* Séguin[2], *Anna* Smirnoff[1], *Jade* Bergeron[1], *Marie-Josée* Morency[2], and *David* Paré[2]

[1]Geological Survey of Canada, Lands & Minerals Sector, Natural Resources Canada, 490 de la Couronne, Québec (QC), G1K 9A9, Canada
[2]Canadian Forest Service, Laurentian Forestry Centre, 1055 du P.E.P.S., P.O. Box 10380, Stn. Sainte-Foy, Québec (QC), G1V 4C7, Canada

**Abstract.** Globally increasing anthropogenic airborne emissions of reactive nitrogen (N) generate several environmental issues that require investigating how N accumulation modifies the N cycle. Tree-ring $\delta^{15}N$ series may help understanding past and current perturbations in the forest N cycle. Although several studies have addressed this issue, most of them were of local scale or based on short $\delta^{15}N$ series. The development of this environmental indicator however would benefit from examining, at the regional scale, the relationships of long tree-ring series with soil N biogeochemical processes. Here we explore these links for tree stands of the oil-sands region in northern Alberta, and the coal-fired power plants region in central Alberta, Canada. We characterize the tree-ring $\delta^{15}N$ trends, the N modification rates and bacterial and fungal communities of soil samples collected in the immediate surrounding of the characterized trees. The dataset suggests that specific soil pH, and N-cycling bacterial and fungal communities influence tree-ring $\delta^{15}N$ responses to anthropogenic emissions, correlating either directly or inversely. Overall, tree-ring $\delta^{15}N$ series may record changes in the forest-N cycle, but their interpretation requires understanding key soil biogeochemical processes. «*In nature nothing exists alone*», Rachel Carson.

## 1 Introduction

Anthropogenic emissions of reactive N in the atmosphere have more than doubled globally since the beginning of industrialization, generating several health concerns, climatic impacts and environmental issues [1]. Releases of various reactive N forms ($NH_3$, $NH_4^+$, NO, $NO_2^-$, $HNO_3$, $NO_3^-$) may change air quality, modify climate dynamics, and contribute to acidification and eutrophication of aquatic and terrestrial ecosystems. Understanding the specific effects on forests requires determining the characteristics of the N species deposited on soils and available

* Corresponding author: martinem.savard@canada.ca

to trees, and understanding processes controlling the historical trends recorded in natural archives such as lakes and trees.

While Canada reports national NOx emissions declining steadily since 2000, emissions in the Province of Alberta remain relatively constant since 2004. With 12% of the Canadian population, Alberta releases 30% of the $NH_3$ and 36% of the $NO_x$ total Canadian emissions. For these reasons, this research focuses on the coal-fired power plant (CFPP) region, southwest of Edmonton, known to generate the highest NOx emissions in Canada, and the surface mining oil sands (OS) region of the Lower Athabasca, north of Fort McMurray in northern Alberta.

With the main incentives of separating natural effects from anthropogenic perturbations and attempting to provide a retrospective look at the forest N cycle in these two regions, Natural Resources Canada and collaborators conducted several multidisciplinary research activities to characterize air, soil, fungi, lakes and trees in exposed forests [2-5]. This proceeding combines isotopic geochemistry, dendrochronology, with soil biogeochemistry, genomics and microbial ecology, and briefly discusses the post-depositional processes modifying the biogeochemical pathways in soils and trees.

## 2 Methods

### 2.1 Collection and preservation of samples

Soil and tree samples were collected during fall, between 2010 and 2017, from three sites in the OS region (S2, SV, SC), and two sites in the CFPP region (PRW, WBR). Site SV in the OS region burned down during the large 2016 Fort McMurray wildfire. Consequently, its soils were only partly sampled and characterized. Its tree-ring series and partial soil data set serve for comparison with results of site C, which show similar soil conditions. Stem sections of three old trees selected at each site to cover the operation and pre-operation periods were collected for dendrogeochemistry, and let to dry out prior to sub-sampling for isotopic analytical work (e.g., [2]). Four radii of the tree stems were cut apart in four perpendicular directions. Once dated, the rings from each radius were dissected into individual growth rings and combined into a given-year sample prior to treatment for removal of resins from ring wood.

Rootlets collected in four directions around trees were separated manually from rootlet prats coated with ectomycorrhizal fungi (EcM). These two types of samples were treated separately (see section 2.5).

Soil samples from the organic layer (F and H horizons) and the mineral soil (A and B horizons) were collected in the north, south, east and west directions, in the immediate surroundings of each selected trees at the two sites. Samples were sieved at 4 mm for organic, and 2 mm for mineral horizons. In addition, pedons were sampled from the L to the C horizons (Table 1). The FHAB horizons near trees were split into three parts for the different analytical treatments: isotopic abundance, and microbial and biogeochemical characterization. For the latter, samples were refrigerated, whereas those collected for isotopic analyses and genomic characterizations of microbial communities were immediately frozen with dry ice and kept frozen until lyophilisation in the laboratory.

**Table 1.** Number of samples analysed per site up to now in the two selected regions of Alberta.

| | **Region** | **Lower Athabasca Oil Sands** | | | **Coal-fired power plants** | |
|---|---|---|---|---|---|---|
| | Site | S2 | SC | SV | PRW | WBR |
| $\delta^{15}N$ | Trees/Period | 1880-2015 | 1900-2017 | 1900-2015 | 1880-2016 | 1925-2016 |
| | tree-ring n | 333 | 195 | 348 | 187 | 120 |
| | Rootlets/ECM | 12/12 | 12/12 | 4/4 | 0/0 | 0/0 |
| | FHAB | 36 | 36 | 12 | 0 | 0 |
| | Pedon/horizons | 0 | 2/36 | 0 | 0 | 0 |
| Microbiome | Bacteria | 64 | 48 | 16 | 0 | 0 |
| | Fungi | 64 | 48 | 16 | 0 | 0 |
| Geochem | FHAB | 64 | 48 | 16 | 48 | 48 |

## 2.2 Separation and δ15N analysis of soil N species

All treatment and isotopic analyses were performed at the Delta-Lab of the Geological Survey of Canada (Québec, QC). The frozen FHAB and pedon samples were lyophilized, sifted, ground and homogenized. The F, H, A and B samples from the four directions were combined into one for a given tree, and used for the characterization of dissolved inorganic N (DIN; $NH_4^+$ and $NO_3^-$) and dissolved organic N (DON). The N species were extracted from the soil samples using water at a pH representative of rain, centrifuged and filtered.

The $NH_4^+$ and $NO_3^-$ concentrations were determined using an ion chromatograph (930 Compact IC Flex, Metrohm). The $NH_4$ was then separated using the diffusion method and trapped as $(NH_4)_2SO_4$ on glass fibre filters ([6]). The $(NH_4)_2SO_4$-containing filters were dried in desiccator and analysed using an elemental analysis – continuous flow – isotope ratio mass spectrometry (EA-CF-IRMS) system (Costech attached to a Delta V; Thermo Fisher Scientific), and the precision of the method was 0.4‰. The $NO_3^-$ was extracted following the chemical method including Cd for reducing nitrate into nitrite, and sodium azide to produce nitrous oxide ($N_2O$; [7]). The produced $N_2O$ was analysed using a pre-concentrator online with an IRMS (PreCon Delta V). The precision of the method was 0.7‰. A sub-sample of lyophilized, centrifuged and filtered soil material served for total dissolved N (TDN) determination using an EA-CF-IRMS system (Costech attached to a Delta V). A mass balance calculation using the proportions and $\delta^{15}N$ values of the TDN and DIN helped determine the final $\delta^{15}N$ values of the DON.

## 2.3 Preparation and biogeochemical analyses of soil samples

The pH of soil samples was measured in a $CaCl_2$ solution [8]. Exchangeable cations were determined by ICP-AES (Optical Emission Spectrometer, Perkin Elmer, Optima) following a Mehlich III extraction. The cation exchange capacity (CEC) was calculated as the sum of exchangeable cations [8]. $NH_4$-N and $NO_3$-N were extracted with a 2 M KCl solution [8] and analyzed by spectrophotometry (QuikChem R8500 Series 2, Lachat Instruments). Dissolved organic N (DON) was analysed on the same samples following oxidation of a subsample with K-persulfate ($K_2S_2O_8$) and boric acid ($H_3BO_4$) at 121°C and 135 kPa in an autoclave which oxidizes all N forms to $NO_3$ [8-9]. Net ammonification and net nitrification were assessed by incubating soil samples under laboratory conditions in the dark for 42 days and comparing pre- and post-incubation values [10]. Total C and N concentrations were determined by dry combustion using a TruMac CNS analyzer (LECO Corporation).

### 2.4 Genomic characterization of microbial communities

Soil microbial communities were characterized using targeted amplicon sequencing of bacterial and fungal phylogenetic marker genes. Briefly, genomic DNA was first recovered from soil samples using the MoBio Power Soil DNA kit. The V4-V5 16S rRNA gene of bacteria and the internal transcribed spacer 2 (ITS2) region of fungi were then amplified by polymerase chain reaction (PCR) using previously published primers [11-12]. Library preparation and sequencing on an Illumina MiSeq platform was performed as recommended by the manufacturer.

Bioinformatic analyses of Illumina sequencing data were performed in QIIME v1.9.0 [13]. The uSEARCH tool ([14]) was first used to merge sequences, cut the primers, and filter low quality sequences. The uPARSE tool ([15]) was then used to dereplicate the sequences, discard singletons, identify chimeras, and group high quality reads into operational taxonomic units (OTUs) using a 97% identity threshold. The taxonomic assignment of OTUs was generated using the QIIME command "assign_taxonomy.py" with Mothur ([16]) as the assignment method, and Greengenes ([17]) and UNITE ([18]) databases as references for the 16S rRNA gene of bacteria and the ITS2 region of fungi, respectively. The generated OTU table was rarefied with QIIME's "single_rarefaction" command so that the samples all contained the same number of reads. Finally, the OTU rarefied table was used in QIIME's "core_diversity_analyses" to generate alpha diversity measures, calculate the beta-diversity between samples, and generate community composition profiles at different taxomomic levels. Further statistical analyses and data visualization were performed in RStudio.

### 2.5 Preparation and isotopic analysis of fungi, rootlets and tree rings

All rootlets, fungi plus rootlets and pre-treated wood of the pooled three-tree samples were analyzed with an EA-CF-IRMS (Costech online with a Delta V) at the Delta-Lab of the Geological Survey of Canada. The ring $\delta^{15}N$ values were averaged out to represent the site signal for a single year. Fungi and fungi plus rootlets were directly weighted and analyzed for their $\delta^{15}N$ values. The precision of the analyses were always 0.3‰ or better. The fungi $\delta^{15}N$ values reported are for fungi plus rootlets (Fig. 1), not for pure fungal material, as the proportion of fungi and wood N per sample are not currently known.

## 3 Results and Interpretation – Local controls on N cycling

### 3.1 $\delta^{15}N$ values of soil N species, fungi (EcM), rootlets and tree-ring series

The H horizons of two pedons at SC and several FHAB profiles at S2, SV and SC in the OS region show average $\delta^{15}N$ values in decreasing order from DON (+5.0‰ ), to $NH_4^+$ (+1.0‰) and $NO_3^-$ (-7.0‰). *It is clear that the $\delta^{15}N$ values in tree ring series will partly depend on the N form rootlet take up.*

In the OS region, the long-term trend during the operation period in the tree-ring $\delta^{15}N$ series of SV and SC increases from -3.1‰ to averages of -1.7 and -1.3, respectively, notably showing an opposite trend relative to the one observed for S2, which decreases from -4.5 down to -6.0‰. Both trends (increasing and decreasing) significantly correlate (directly and inversely) with NOx emissions in the OS region. Examination of individual trees at S2 analyzed for the 1995-2015 period shows that one of the trees has $\delta^{15}N$ values up to 3‰ higher than the values for the other specimens. In the CFPP region, the preliminary results for two trees at site WBR indicate opposite $\delta^{15}N$ response after 1994: from -3.5 up to an average of -2.5‰ (tree 02), relative to a decrease from -3.5 down to -4.5‰ (tree 01). The series at site PRW show a step-down plateau after 1938, from -3.2 to an average of -3.9‰, followed by a decreasing trend after 1962 down to -5.3‰. The long-term trend at this site shows a strong inverse correlation with the CFPP

acidifying emissions. *The tree-ring $\delta^{15}N$ trends triggered by the OS and CFPP operations are rising at acidic sites, but declining at less acidic sites. These observations suggest that the control on the $\delta^{15}N$ values of N taken up by trees is not regional but local.*

At the time of writing this proceeding, the produced $\delta^{15}N$ values for the EcM of all studied sites always exceed these of tree rings. An interesting feature is that the average EcM $\delta^{15}N$ value for SC EcM is clearly higher than the one for S2. *These observations support previous explanations that EcM retain heavy N and release light forms for their hosts [19], and suggest that the EcM $^{15}N$-$^{14}N$ fractionation differs between these two sites.*

### 3.2 Soil biogeochemistry

In the OS region, S2 has distinct biogeochemical properties relative to SV and SC namely a higher pH and CEC linked with higher concentrations of Ca and Mg, and less total N and available P. A higher rate of net nitrification and a lower rate of ammonification were also found in S2 relative to SC and SV soils. In the CFPP region, WBR has higher soil pH, CEC and concentrations of exchangeable K, Ca and Mg, relative to PRW, especially in mineral horizons. Similar to the OS region, the higher pH and CEC found at WBR were correlated with lower concentrations of total N and available P along with a lower rate of net ammonification. Soil pH in the CFPP region is considerably higher (average pH=6.01) than that in the FMM region (average pH=4.93), except for the pH at S2 (average pH=5.89), which is similar to that of the CFPP region. *These inter- and intra-site differences in soil pH can explain the varying ability of microorganisms to produce N forms during laboratory incubations, and suggest lower rates of nitrification but higher rates of ammonification in the most acidic settings of the studied sites (SC and SV).*

### 3.3 Soil microbiome

Similar to soil biogeochemical properties in the OS region, microbial communities were generally distinct in S2 when compared to SV and SC. Samples from S2 were separated from samples of sites SV and SC on the first axis of the Principal Coordinate Analyses (PCoA) ordination plots for both bacterial and fungal communities, while the soil horizon had a lower effect. The bacterial alpha diversity was generally higher in S2 than in SV and SC, especially in the mineral horizons, while the opposite trend was observed for fungi. The bacterial community composition at site S2 was characterized by higher relative abundances of the phyla Chloroflexi, Proteobacteria and Nitrospirae, while the phylum Actinobacteria was detected at higher relative abundances at sites SV and SC. These two sites were also characterized by the presence of groups of Acidobacteria commonly associated with lower pH environments. Differences in fungal community composition were mainly detectable at lower taxonomic levels. As an example, the ECM fungal genus *Piloderma* had a higher relative abundance in SV and SC than in S2, while the saprotroph *Mortierella* was mostly found in S2. The characterization of the microbial communities in the CFPP region is ongoing. *Based on the data currently at hand, local soil conditions appear to have controlled the distinct bacterial and fungal communities in soils surrounding trees at the studied sites.*

## 4 Preliminary Interpretation of integrated data - Fate of airborne N

Previous publications have determined the $\delta^{15}N$ values of open-field particulates at various distances from the OS open-mining operations [20]; the summer averages for $NO_3^-$ and $NH_4^+$ are -4.0 and -0.2‰, respectively. The sole addition of N species with such signals cannot readily explain the suite of trends observed in the OS region. Similarly, $HNO_3$, p-$NO_3^-$ and

$NH_4^+$ samples simultaneously collected in plumes downwind from the CFPPs ([3-4]) with summer average values of +5.5, +5.4‰ and -1.6‰, respectively, could explain the increasing trend observed in one tree, but could not account for the decreasing trends in the 4 other trees. Given that the emissions in the two regions correlate with the various tree-ring isotopic trends, it seems that locally controlled fractionation processes triggered by anthropogenic N inputs are responsible for the observed changes.

The contrasting rates of microbial ammonification and nitrification between more and less acidic soils support this hypothesis. The acidic SV and SC showing the increasing $\delta^{15}N$ trends have higher ammonification, but lower nitrification rates than less acidic soils (S2, PRW), the consequence being that more $^{15}N$-rich $NH_4^+$ and less $^{15}N$-depleted $NO_3^-$ are available in acidic soils. Moreover, in such soils, EcM may take up more amino acids (in DON; [21-22]), the most $^{15}N$-rich N form analysed here (Fig. 1).

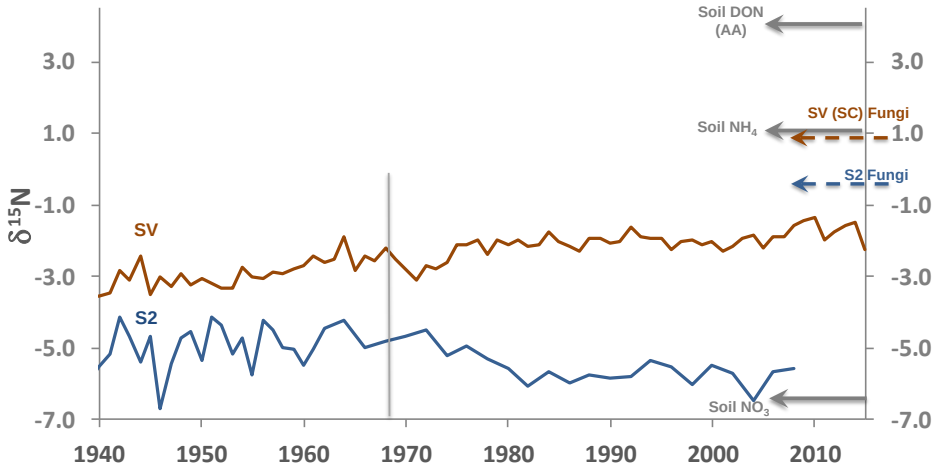


**Fig. 1.** Average tree-ring $\delta^{15}N$ results (‰) for sites SV and S2 as a function of time, and compared with bulk average fungi values (stippled arrows) for each site (without correcting for presence of wood in samples), and preliminary values for soil N species (solid arrows). The vertical line indicates the onset of the OS mining operations.

Overall, the integration of the tree-ring, root, EcM and soil isotopic data along with the soil microbial N-transformation rates, pH and microbiome results indicate that the anthropogenic N emissions trigger distinct responses at the various sites, locally controlled by existing soil conditions prior to anthropogenic N accumulation. The strong correlations of tree-ring $\delta^{15}N$ trends with anthropogenic N emissions in the studied regions imply that the tree-ring series have the potential to record past changes in soil N availability.

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